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INTRODUCTION

This report covers research activities during the period December 1, 1970, through May 31, 1971, for the project entitled "Environmental Microbiology as Related to Planetary Quarantine." These studies were conducted by the Division of Environmental Health, School of Public Health, at the University of Minnesota under the auspices of the National Aeronautics and Space Administration and Lawrence B. Hall, Planetary Quarantine Officer.

This is the sixth semiannual report of progress on NASA project NGL 24-005-160. A limited number of earlier reports on this project are available for those who do not have a complete set but who wish to obtain all of our procedures and results in a given area.

Studies have been underway for more than a year to determine the effect of relative humidity on the survival of microbial spores at 45, 60, 75 and 90°C. In these studies we are attempting to evaluate the survival characteristics at a range of relative humidities between 0 and 100% measured or calculated at the test temperature. In previous studies at 45 and 60°C we were not able to reproduce the parabolic curve that was originated by Murrell, Scott (1957, 1966) and Angelotti et al.(1968) when the logarithm of the D-value was plotted as a function of water activity (A_w) with the maximum D-value at an A_w of between 0.2 and 0.8 and minimum D-value at an A_w between 0 and 1.0. In our studies at 45 and 60°C the maximum D-value occurred at the lowest relative humidity evaluated. The data we have obtained at 90°C suggest that the maximum D-value at this temperature is at or below a relative humidity of 15%. These results suggest that the relative humidity (RA_w) for maximum survival of Bacillus subtilis var. niger spores is not constant but is a function of temperature. As part of our future work it seems desirable that we determine whether or not this phenomena which has been observed for Bacillus subtilis var. niger spores also exists for other species of microbial spores.

I. J. Pflug

SURVIVAL OF MICROBIAL SPORES UNDER SEVERAL TEMPERATURE AND HUMIDITY CONDITIONS

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INTRODUCTION

In previous progress reports we have described and reported the results of experiments carried out to measure the survival rates of Bacillus subtilis var. niger on surfaces at 22, 45 and 60°C as a function of the relative humidity of the surrounding environment. We have also reported the results of experiments to determine the destruction characteristics of spores suspended in several different concentrations of liquids commonly used for the storage of spores, all at temperatures of 45, 60, 75 and 90°C.

This report is concerned with activities carried out to determine the effect of temperature and relative humidity and includes extensions of both of the previous studies. Results are reported on experiments carried out in a controlled environmental chamber at temperatures of 75 and 90°C (this work was referred to in Progress Report #5). These experiments will complete the series of studies to measure the survival rates of Bacillus subtilis var. niger on surfaces as a function of the relative humidity of the surrounding air. Results on an extension of the investigation of destruction characteristics of spores suspended in solutions of various water activities (A_w) are also reported. Specifically, survival characteristics are reported for Bacillus subtilis var. niger (AAOE) spores which were suspended in sucrose solutions at 90°C (with water activities of 0.99, 0.90 and 0.85).

OBJECTIVES

The objectives of these experiments are: 1) to determine the survival of spores on surfaces at several temperature and relative humidity conditions and 2) to determine the survival of spores in a liquid system at several water activity conditions.

EXPERIMENTAL PROCEDURE

Sucrose and glycerol - Tests GS0281A, GS0288A, GS0293A, GS1046A,B,C, GS1067A,B,C and GS0342A,B,C

The survival of Bacillus subtilis var. niger spores AAAA and AAOE suspended in solutions of both glycerol and sucrose at calculated A_w's of .99, .90 and .85 were evaluated at 45, 60, 75 and 90°C. Table I.I summarizes the information concerning the solutions which were prepared (using the data of Scott[1]) and used in these experiments.

Table I.I

Concentrations of Glycerol and Sucrose Used to Obtain Water Activities of .99, .90 and .85

A_w at 25°C	Glycerol molality percent[a]		Sucrose molality percent[a]	
.99	0.554	4.85	0.534	15.45
.90	5.57	33.90	4.11	58.45
.85	8.47	43.80	5.98	67.15

[a]percentage by weight

The solutions were prepared aseptically using reagent grade sucrose, Difco glycerol and sterile distilled water. The solution was dispensed into a series of sterile 25 x 150 mm screw-cap test tubes (17.5 ml per tube) and refrigerated until used. A number of tubes were checked for viable microorganisms using Trypticase Soy Broth and no growth was found. Ten µl of spore suspension were added to the liquid in each tube; the contents were then mixed on a Vortex mixer. The final target concentration of the spores was about 2×10^6 spores per ml. After the spores and substrate were thoroughly mixed, the tubes were placed in a circulating water bath at the test temperature ±0.5° for varying intervals of time.

Four tubes were randomly selected at time zero to serve as controls. Four randomly selected tubes were removed and evaluated at each time interval. After removal from the water bath the tubes were cooled in an ice water bath for four minutes. Two one-ml samples were taken from each tube, diluted in buffered distilled water and plated in duplicate using Trypticase Soy Agar. Plates were incubated at 32°C for 48 hours and then counted. The data were analyzed using the guidelines presented in Progress Report #3, except that in some cases we utilized counts below 20 colonies.

Effect of temperature and relative humidity at 75 and 90°C

These experiments are a continuation of the overall effect of temperature and

[1]Scott, W.J. "Water Relations of Food Spoilage Microorganisms." Advances in Food Research, 7, 83-127, 1957.

humidity progress. To carry out experiments at 75 and 90°C, it was necessary to have a new experimental system. The system that we have developed and are using in these experiments utilizes an environmental chamber with horizontal airflow where temperature can be controlled up to 100°C and relative humidity between 15 and 90%. The basic environmental chamber was equipped with a door 97 cm x 102 cm. Opening and closing this large door to insert or remove samples alters the conditions inside the environmental chamber for several minutes following the operation. Since varying conditions may cause either a wetting or drying of samples in the environmental chamber, the glass door unit was modified by installing a plastic door unit equipped with two hand-hole units. The inside of the environmental chamber was modified so that special hanging units could be moved in through the hand-holes, put in place quickly and subsequently removed through the hand-holes.

Experiments were carried out in the following manner. Stainless steel planchets, 1 x 2 inches, with a 3.5 mm diameter hole in one end were inoculated with 10^6 spores of Bacillus subtilis var. niger (AAOE). The inoculated planchets were equilibrated for 18-24 hours in the clean room. The planchets were then placed in covered polyethylene containers and carried to the environmental chamber.

Insertion of room-temperature planchets into an atmosphere of 75 or 90°C and 75% relative humidity produces condensation on the surface of the planchet. Therefore it was necessary to preheat the planchet prior to placement in the chamber. The sequence of operations was as follows. The planchets were: 1) placed on the hangers, 2) moved into a dry-air oven that was 10°C below the treatment temperature (the oven was adjacent to the environmental chamber), 3) held in the dry-air oven for three minutes and 4) transferred to the treatment chamber where they were placed with the spore inoculum facing away from the direction of the air flow. Each hanger held the three planchets that were subjected to each time-temperature-humidity condition. The location of the hanger in the chamber was randomly determined.

After the appropriate length of time the planchets were removed from the chamber and each planchet was placed in a sterile 125 ml Erlenmeyer flask that had been cooled in an ice bath. The flask containing the planchets were carried to the clean room where the planchets were assayed for survivors using NASA standard procedures. Two samples from each planchet were plated in duplicate using Trypticase Soy Agar. Incubation was carried out at 32°C for 48 hours.

RESULTS AND DISCUSSION

In Figures 1.1, 1.2, 1.3 and 1.4 are shown the survivor curves obtained in sucrose at 45, 60, 75 and 90°C as a function of the three water activities evaluated,

0.65, 0.90 and 0.99. In all cases as the water activity decreased microbial survival increased. As temperature increased from 45 to 60 to 75 to 90°C these effects were more pronounced. Further analyses of these data are underway.

In Figures I.5, I.6, and I.7 are shown the survivor curves obtained at 90°C using the environmental chamber system. These data are summarized in Table I.8 where we show the times for a two and a four log reduction in counts as a function of relative humidity. These studies are still underway. The results of the analyses of these data along with the new data will be presented in the next progress report.

FUTURE WORK

Studies that are currently underway will be completed. These include:

1. Destruction rate studies of Bacillus subtilis var. niger spores in glycerol and sucrose at .85, .90 and .99 water activities.

2. The effect of temperatures of 45, 60, 75 and 90°C and a range of relative humidity conditions on the destruction rate characteristics of Bacillus subtilis var. niger spores.

When these studies have been completed we will proceed to study the survival of additional species of microorganisms at several different temperature and relative humidity conditions.

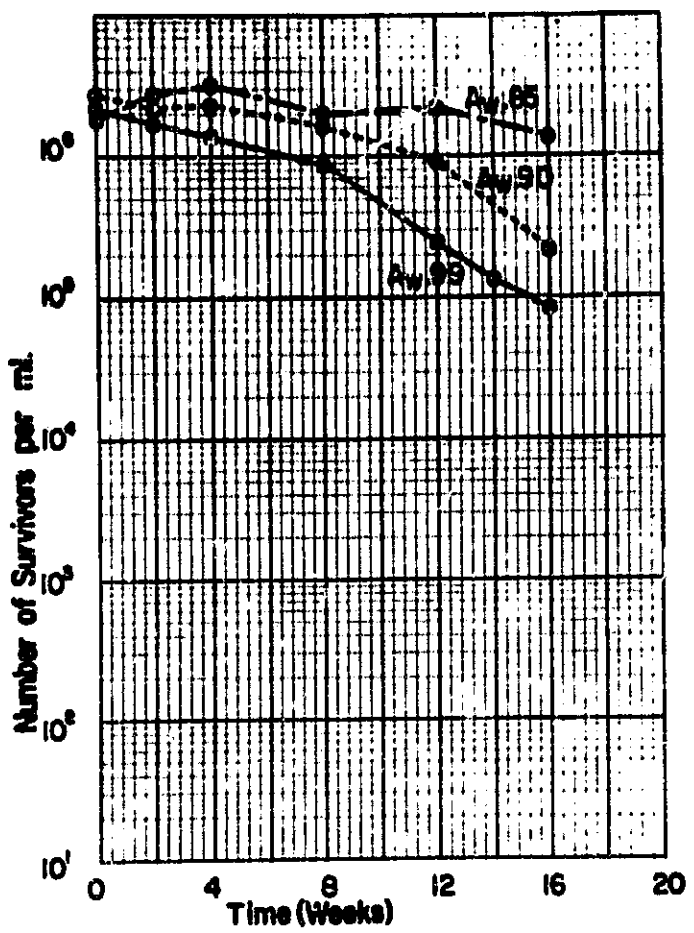

Figure 1.1 - Survivor curves for AAOE spores in sucrose solutions at 45°C

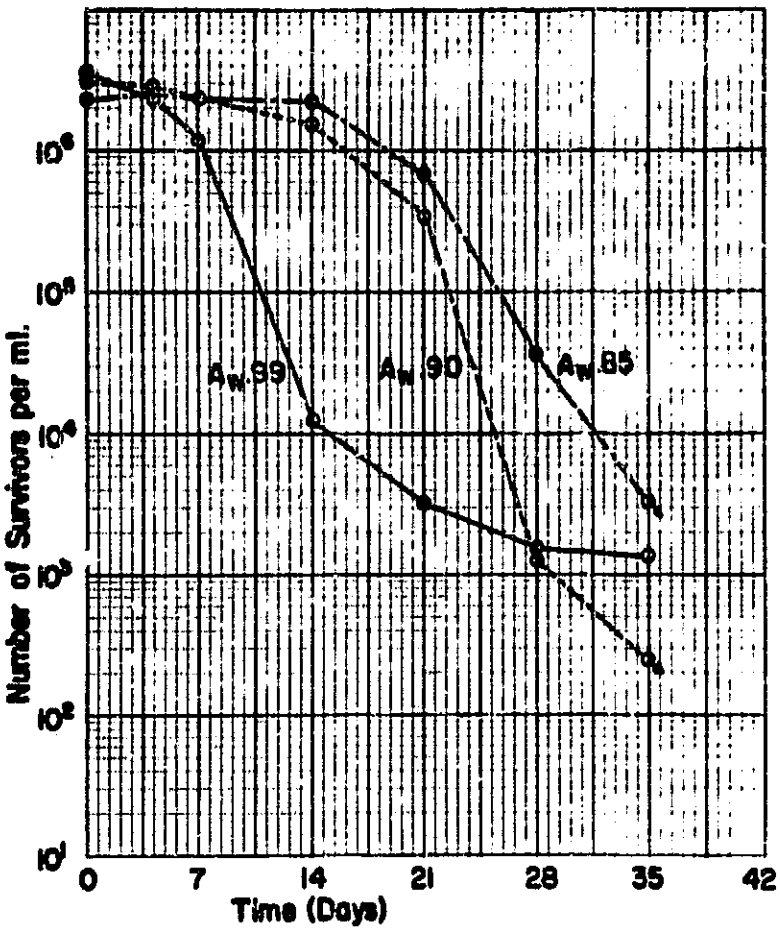

Figure 1.2 - Survivor curves for AAOE spores in sucrose solutions at 60°C

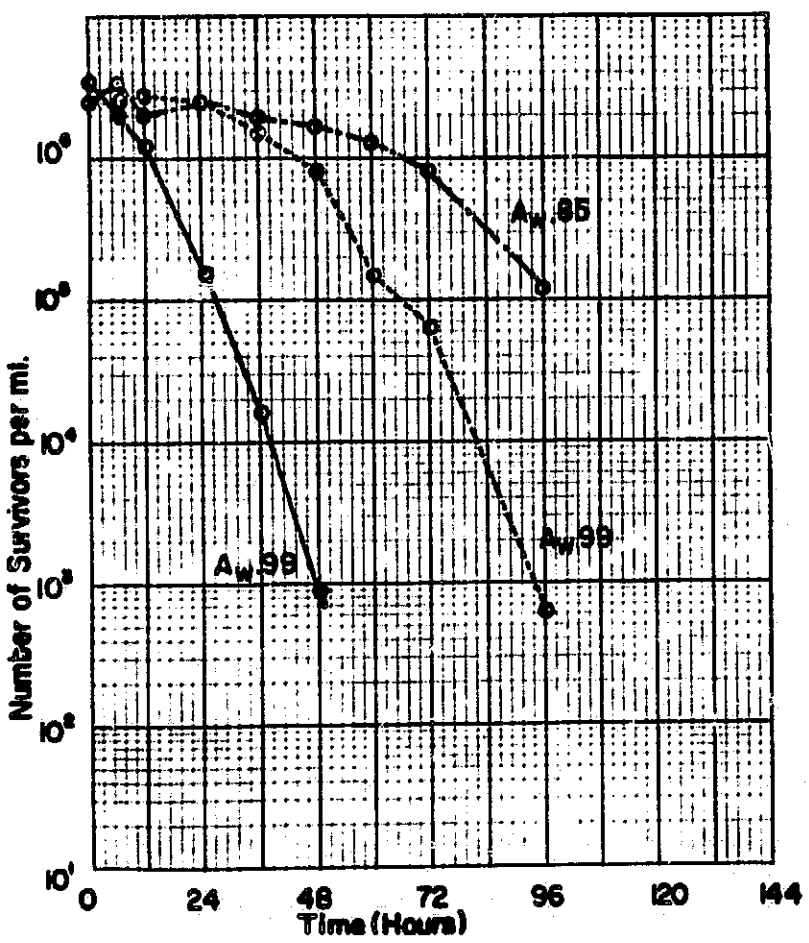

Figure 1.3 - Survivor curves for AAOE spores in sucrose solutions at 75°C

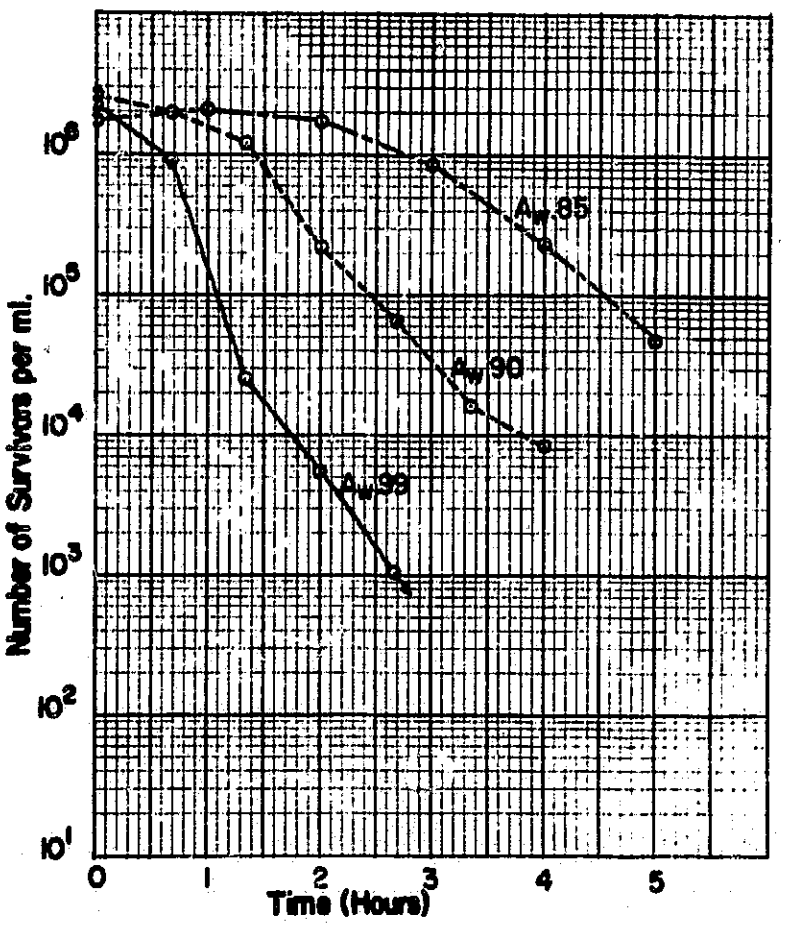

Figure 1.4 - Survivor curves for AAOE spores in sucrose solutions at 90°C

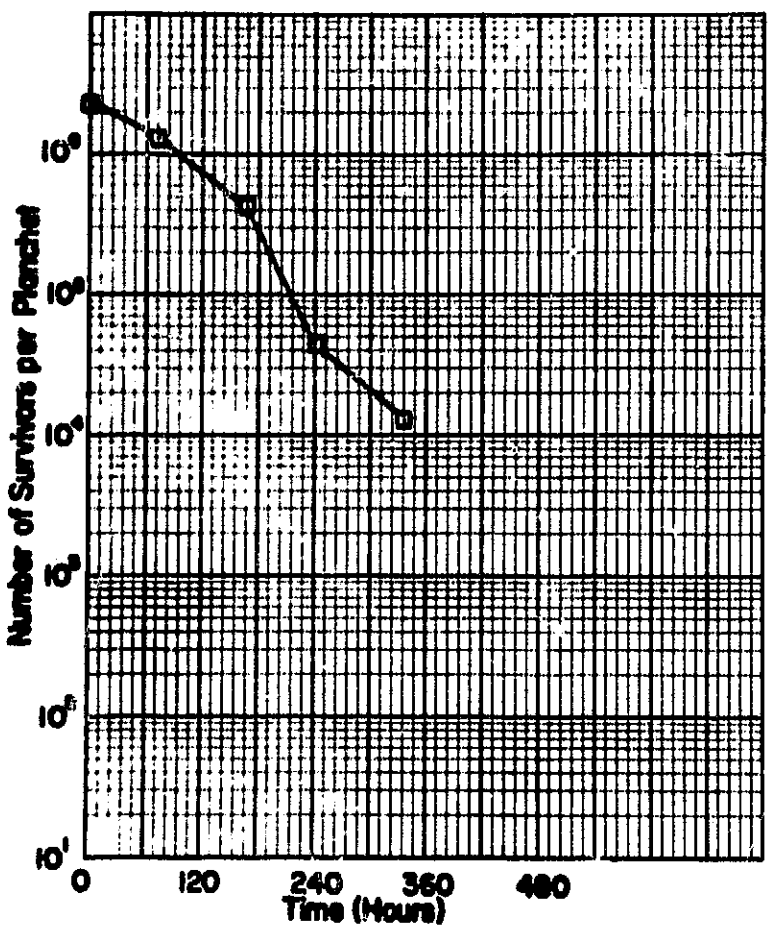

Figure 1.5 - Survivor curve for AAOE spores at 90°C, 15% RH

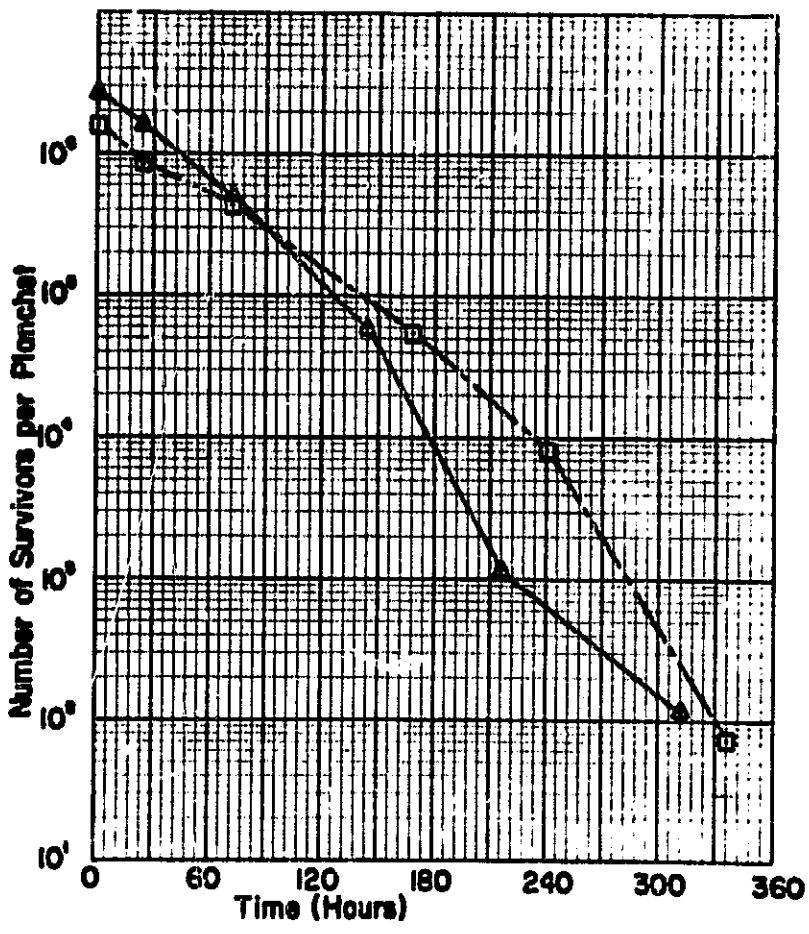

Figure 1.6 - Survivor curve for AAOE spores at 90°C, 35% RH

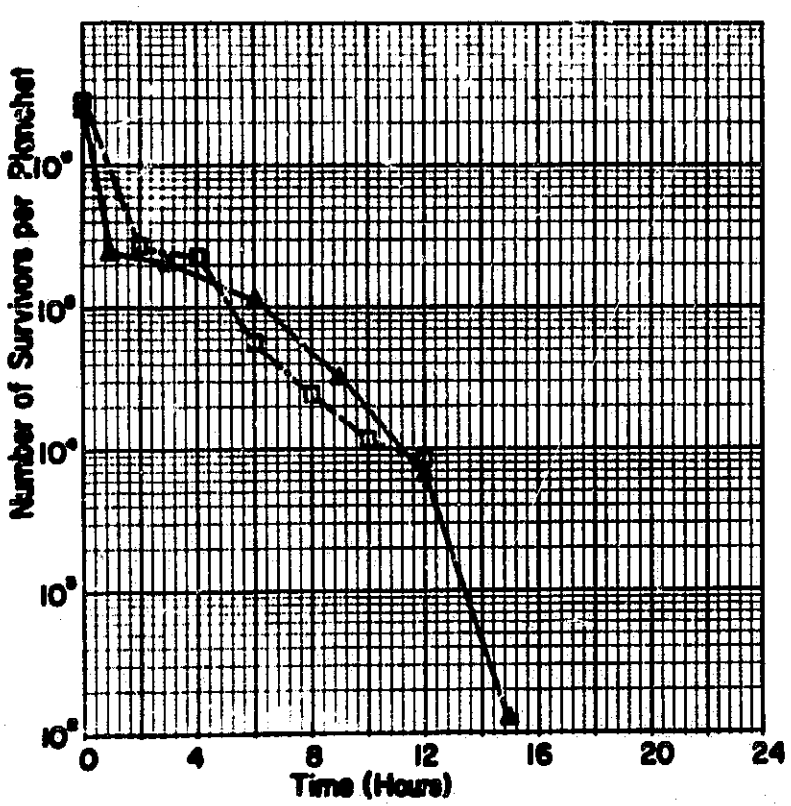

Figure 1.7 - Survivor curve for AAOE spores at 90°C, 75% RH

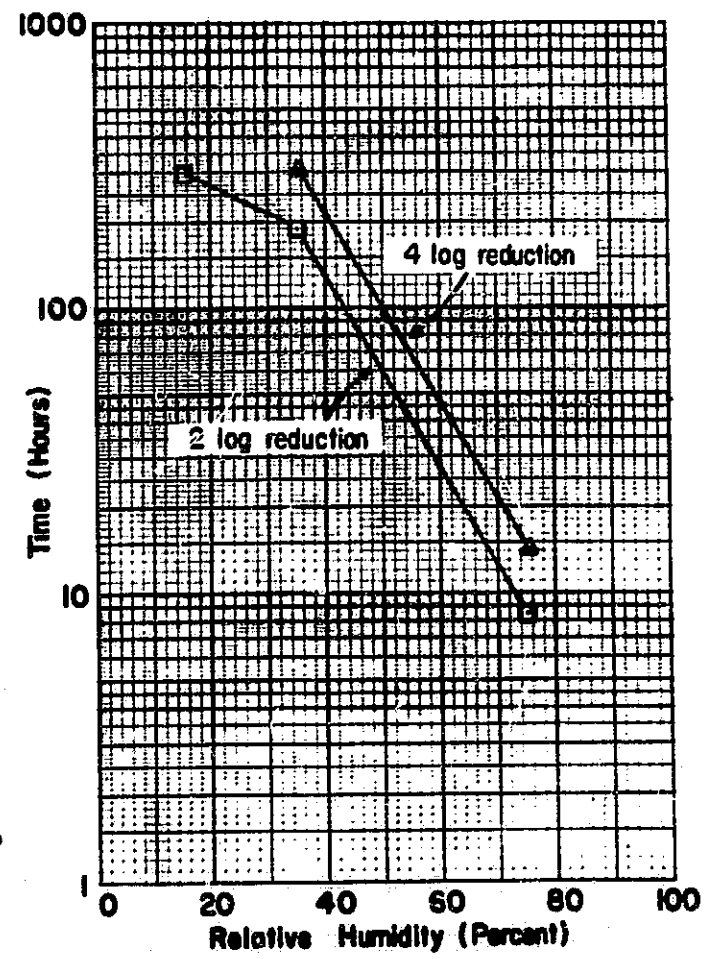

Figure 1.8 - Time for a 2 and 4-log reduction in spores at 90°C versus relative humidity

DETECTION OF LOW LEVELS OF MICROBIAL CONTAMINATION ON SURFACES BY CHEMICAL APPROACHES*

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INTRODUCTION

Qualitative and quantitative determinations of bacterial life have been performed for many years. The procedures used have relied on the growth of the microorganisms on suitable media. These procedures require specific conditions and a minimum period of time which is usually 24 hours. However, a method has been developed for the rapid detection of small quantities of microbial cells by chemical techniques. This method utilizes the quantification of adenosine triphosphate extracted from bacterial cells on thin-layer chromatography. The evaluation is performed directly on thin-layer chromatograms with the use of a microscope photometer to measure the intensity of the emitted light produced by exciting adenosine triphosphate with ultraviolet light at 360 nm. The major advantage of this method over older techniques is that the actual analysis of the microbial cells requires about 45 minutes.

The method involves a chemical procedure and is based on the presence of adenosine triphosphate (ATP) in the cell structure of the microbes. Adenosine triphosphate is a labile, energy-rich compound found in all living organisms and is generally accepted as an indicator for the presence of life.[3,5] The quantities of adenosine triphosphate measured under specific conditions are about 1×10^{-8} micrograms. The method incorporates thin-layer chromatography, known for an excellent sharpness in the separation of compounds, high sensitivity and rapid analysis, along with ultraviolet light and the microscope photometer. The evaluation of ATP is accomplished by direct spectrophotometric analysis on thin-layer plates. This method has greater potential for rapid characterization of compounds than any other method presently available.

ANALYTICAL METHOD

In this described method, microbial cells are exposed to a solvent system on thin-layer chromatographic plates and are subjected to direct chemical analysis without mechanical cell wall rupture. The separation process takes place in an airtight chamber. Using ascending chromatography the compounds are extracted and separated on purified MN 300 cellulose layer. The developed chromatograms are

*This manuscript summarizes the activities of the project and is to serve as the final report of the project which was closed out May 31, 1971.

examined visually in ultraviolet light at 360 nm and then evaluated using a microscope photometer.

The intensity of the emitted light from ATP is increased by coupling with a fluorochrome compound, fluorescein, which is added to the solvent system. This process increases the sensitivity of the method.

The thin-layer chromatographic material used in the development of the method and in the tests was MN 300 cellulose without a binder supplied by Macherey, Nagel and Co., Duren, Germany. Analytical grade adenosine triphosphate was obtained from Mann Research Laboratories, Inc., New York, New York. Escherichia coli were prepared according to standard methods. Fluorescein was obtained from Merck and Co., Inc. The solvent system for the thin-layer separation consisted of n-butanol/acetone/acetic acid/5% ammonium hydroxide/water in the ratio of 4.5:1.5:1:1:2.[8] Organic solvents were obtained from Eastman Kodak Co.

EXPERIMENTAL PROCEDURE

The chromatoplates may be prepared ahead of time and stored in an airtight desiccator. In this preparation, purified cellulose powder was used. Fifty grams of the powder were suspended in 500 ml of the solvent system in a glass stoppered Erlenmeyer flask, shaken for 30 minutes and then filtered. The solid residue was rinsed with n-butanol, and dried in a vacuum until ammonia was removed.[7]

For the preparation of the chromatoplates, 15 grams of the purified cellulose were suspended in 95 ml demineralized glass distilled water and homogenized for 45 sec. A measured quantity of the prepared cellulose water slurry was applied onto thoroughly cleaned glass microscope slides and the layers were made even by gentle tilting. The even surface of the layer is a very important factor in the direct scanning procedure. The coated plates were allowed to dry at room temperature overnight when activated for 15 min. at 110°C and stored in a sealed desiccator.

Before use, the prepared chromatoplates were chromatographically cleaned by means of a preliminary development in the solvent system. The solvent was allowed to run the entire length of the plate. The chromatoplates were then dried at room temperature in a glove box with relative humidity maintained below 5%. A dull-yellow colored band visible in daylight and brightly fluorescent in ultraviolet light appeared at above 75% distance from the starting line. The area used for the adenosine triphosphate standard solutions and microbial analysis was below this yellow line.

An aqueous standard solution of adenosine triphosphate was prepared by weighing out the crystalline ATP compound on an electrobalance and diluting it with demineralized glass distilled water to a concentration of 1 microliter/microgram and then

diluting 1:1,000 and 1:10,000. Escherichia coli and yeast cells were transferred with a glass loop directly to a tarred, light-weight aluminum foil weighing pan on an electrobalance and weighed in 10 microgram quantities. The weighed materials were transferred together with an aluminum support to a volumetric flask and diluted with demineralized water and mixed thoroughly. The 1 microliter = 1 microgram concentration of the prepared suspension was then diluted 1:1,000.

Analysis Technique

Owing to the marked influence of humidity on the appearance of adenosine triphosphate fluorescence on thin-layer chromatograms, the analysis procedure was conducted in a glove box in which the relative humidity was maintained below 5%. The standard ATP solution and suspensions of E. coli and yeast cells were immediately spotted with a 10 microliter syringe (graduated in 0.1 microliters) on geometrically predetermined spots five millimeters from the bottom of the chromatoplate. After evaporation of the water in which the materials were suspended, the group of chromatoplates were rearranged on a glass multi-plate holder and placed in a chromatography chamber containing the solvent system. The ascending chromatographic action was discontinued when the solvent line was just below the dull-yellow line formed in the pretreatment process. The distance between the solvent front and the application spots was approximately 4.5 cm and the developing time was about six minutes. The developed chromatograms were dried in the low relative humidity glove box with an air stream blown through the air inlet for twenty minutes. The chromatograms were then evaluated directly in a Zeiss Microscope Photometer, Model MPM. In this unit the chromatograms were exposed to ultraviolet light from an Osram HBO-100 W/2 Source, by focusing on the blank area and scanning lengthwise from the spotting point and to the solvent end line. The intensities of the emitted light from the excited adenosine triphosphate were registered with a Zeiss microscope photometer display unit and Honeywell recorder. The following experiments were conducted:

1. Fluorescein

   Five concentrations, 10, 5, 1, .5 and .1 ppm, of fluorescein in the solvent system were prepared.

   a. Four groups of twelve activated micro thin-layer plates were developed in each solvent system containing the different fluorescein concentrations in full-length.

   b. The 0.1% solution of fluorescein was spotted on the dry chromatoplates from experiment 1.a and developed in solvent systems. The solvent

system was allowed to travel 4.5 cm from the starting line to the yellow line.

2. Adenosine triphosphate (ATP)

An aqueous standard solution of ATP was chromatographed on chromatographically precleaned chromatoplates in experiment I.a, where the solvent system contained different fluorescein concentrations. The quantities of adenosine triphosphate used were from 1×10^{-2} to 1×10^{-10} micrograms.

3. Escherichia coli and yeast cells

a. The aqueous suspension of E. coli and yeast cells was chromatographed on precleaned chromatoplates with a fluorescein concentration of 0.5 ppm in the solvent system.

b. The same procedure was used with ATP added to the aqueous suspensions of E. coli and yeast cells.

RESULTS

The procedure for treating bacterial cells and the evaluation of the quantities of ATP using ultraviolet light and the microscope photometer were repeated numerous times with excellent precision.

Figure 2.1 shows the developed micro thin-layer chromatogram in ultraviolet light at 360 nm from ATP (1) and E. coli (2) chromatographed in the solvent system with a fluorescein concentration of 0.5 ppm. The extracted ATP from E. coli and standard ATP shows up in the ultraviolet light as bright yellow spots on a weakly fluorescent background.

Bacterial cells were also tested after being washed several times with normal saline in order to remove medium constituents. Chromatograms with the washed microorganisms produced patterns similar to those shown in Figure 2.1.

In experiment I different concentrations of fluorescein were used to establish the influence of the material on the background quality of thin-layer plates. Examination of the developed chromatoplates in ultraviolet light at 360 nm revealed only one yellow line at about 75% of the distance from the starting line to the end of the plate. The area between the starting line and the bright yellow line was weakly fluorescent. It was also shown that the fluorescein alone does not form any spots in the traveling distance but moves with the solvent end line. This test was conducted by chromatographing 0.1% fluorescein solution on the area of the precleaned chromatoplate between the starting point and the bright yellow line.

Adenosine triphosphate was chromatographed on precleaned chromatoplates from experiment I.a in order to determine the concentration of fluorescein that produces

maximum light emission. Quantities of ATP chromatographed for this purpose ranged from 1×10^{-3} to 1×10^{-8} micrograms. The results are shown in Figure 2.2. The curve was prepared by plotting maximum readings of one ATP quantity chromatographed with different amounts of fluorescein versus fluorescein concentration. The curve shows that the optimum concentration of fluorescein added to the solvent system would be 0.5 ppm.

Quantities of ATP ranging from 1×10^{-2} to 1×10^{-10} micrograms were chromatographed with 0.5 ppm fluorescein concentration added to the solvent system in order to prepare a standard curve. The standard curve for ATP measured with the microscope photometer is presented in Figure 2.3.

The readings of the prepared chromatograms with ATP were made up to a concentration of 1×10^{-8} micrograms. Lower concentrations were omitted because of high noise level from contamination of airborne particles. It became apparent that complete control of contamination from airborne particles and other conditions would be required in order to increase the sensitivity of the method.

In experiment 3, E. coli and yeast cells were chromatographed on the prepared chromatoplates with the solvent system containing 0.5 ppm fluorescein. Figure 2.4 shows the data obtained in this work.

The results according to the ATP standard curve and curves obtained from E. coli indicate that the number of cells detected would be about ten if the weight of one E. coli cell is 1×10^{-12} grams. This study was performed with bacterial cells in their growth phase, however, more work is necessary to obtain greater accuracy because of the many factors which influence the quantity of ATP in the cells.[2,1]

Adenosine triphosphate chromatographed along with E. coli or yeast cells demonstrated linear increases as shown in Figure 2.5. It was observed that the range limits of all experiments conducted followed Beer's Law.

Discussion

Many chemical compounds fluoresce when exposed to ultraviolet light at 360 nm. The Zeiss Microscope Photometer, Model MPM is equipped with the filter monochromator which permits the operator to select the optimum light intensity of the emitted light in a very narrow range specific to certain compounds and to calibrate the instrument against the standard material. In this work the emitted light from ATP was measured at 444 nm with a programmed setting of the filter system of the unit.

One of the most important advantages of this method for quantification

of microbial cells is the small amount of time necessary for analysis. This allowed many repeat analyses of all the experiments discussed. Each point on the curves shown in Figures 2.2, 2.3, 2.4, and 2.5 indicates an average of six chromatograms from different preparations. The precision of the method was found to be ±1%.

The reproducibility of direct evaluation with the microscope photometer was also checked by scanning the same spot several times after a definite length of time. It was found that the exposure time to UV light must be limited and standardized. In a long exposure time the light energy is transformed into heat energy and there can be loss of the emitted light quantity. Each evaluation in the microscope photometer requires only 2 to 3 minutes. There were no noticeable changes on repeated scanning after 5 minutes, but there was a noticeable loss after 10 minutes.

A very important factor in micro thin-layer chromatography on fluorescent compounds is humidity. It was noticed in the early phase of the study that high relative humidity depressed the fluorescence. It is known that high moisture content in the thin-layer bed will delay fluorescence.[4,6,9] It was established that relative humidity maintained below 5% produced good results in 15 minutes with vigorous air movement.

An important factor in thin-layer chromatography is the sample application. The precision and accuracy of the delivered quantities of material on the thin-layer plates were improved by using a special stand with millimeter scales to hold the 10 microliter syringe in a rigid position. The precision of the application simplifies the scanning procedure in the microscope photometer. When the objective is focused on the application area, scanning is performed lengthwise along the thin-layer chromatogram until the yellow line is reached.

For ultimate sensitivity the microscope photometer requires a high quality of thin-layer chromatographic plate with special surface characteristics and a high degree of purity of the thin-layer material. In order to fulfill those requirements, the following steps should be considered:

1. Purification of the thin-layer material
2. Improving the surface quality of the plate
3. Preparing the chromatographic plates to provide even thickness
4. Improving the sample application
5. Developing aids that will make it possible to prepare uniform thin-layer plates rapidly
6. Studying of factors that affect the development of the chromatogram such as humidity and contamination from airborne particles.

Most of these requirements were fulfilled in the studies described. It is necessary to take these steps in order to increase the sensitivity of the method,

to eliminate the noise level and get reproducible background readings in the microscope photometer. As described above, it was found that the glove box can solve this problem satisfactorily. As discussed previously, only one step in the above procedure was conducted in the glove box which served as a miniature laboratory with controlled humidity, temperature and clean air. The manual work was performed through the glove ports while wearing thin rubber gloves. The glove box, measuring 1.5 x 1 x .8 meters was also equipped with an air inlet and outlet in order to replace the air containing toxic solvent vapors. The contaminated air was exhausted after the chromatograms were removed and then the glove box was prepared for the next run.

The described method for the evaluation of trace quantities of ATP can be used for other fluorescent and nonfluorescent compounds depending upon the versatility of the Zeiss Microscope Photometer. It is believed that such a procedure could be utilized in the exploration of space where identification of the presence of life is necessary and chemical changes in living cells in outer space is possible. Likewise, the method can be adopted to various situations where trace quantities of fluorescent materials may be an indication of cell change.

SUMMARY

A method has been described for the rapid detection of small quantities of microbial cells by a chemical approach. The direct scanning of the adenosine triphosphate on thin-layer chromatoplates offers results within 45 minutes. More studies are required in order to enumerate the cells, because ATP content in microbial cells is a variable factor and depends upon the environment in which the cells exist. It is planned that with additional studies the developed method would also serve to determine the presence of microoganisms on surfaces.

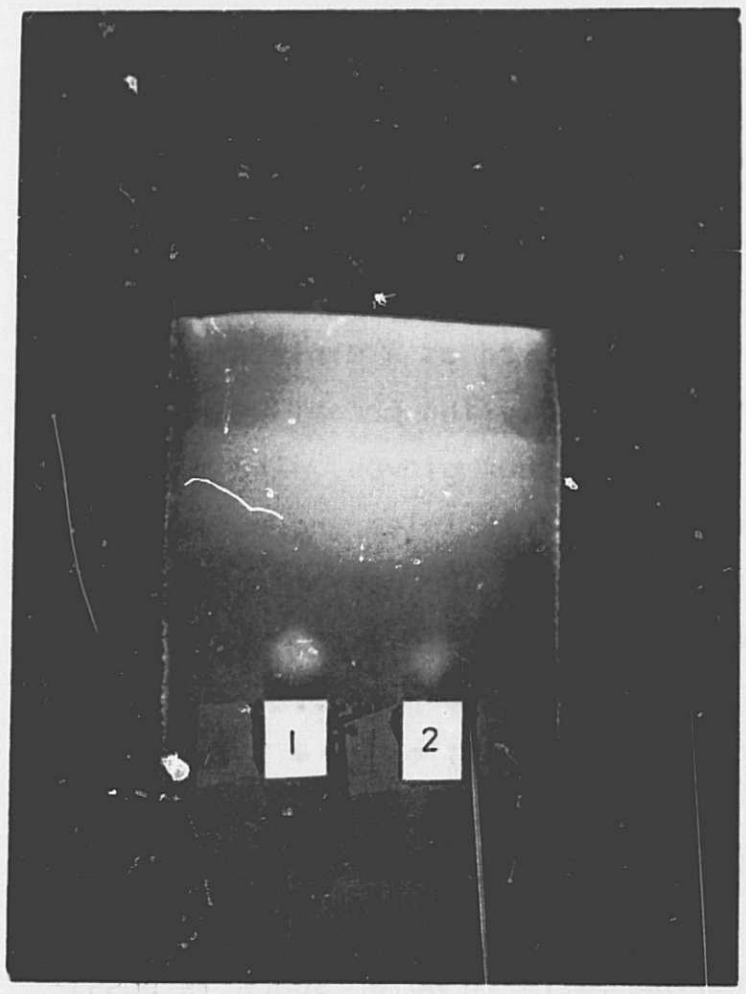

Figure 2.1 - Thin-layer chromatogram on microscope slide photographed in ultraviolet light at 360 nm. 1-ATP, 2-E. coli.

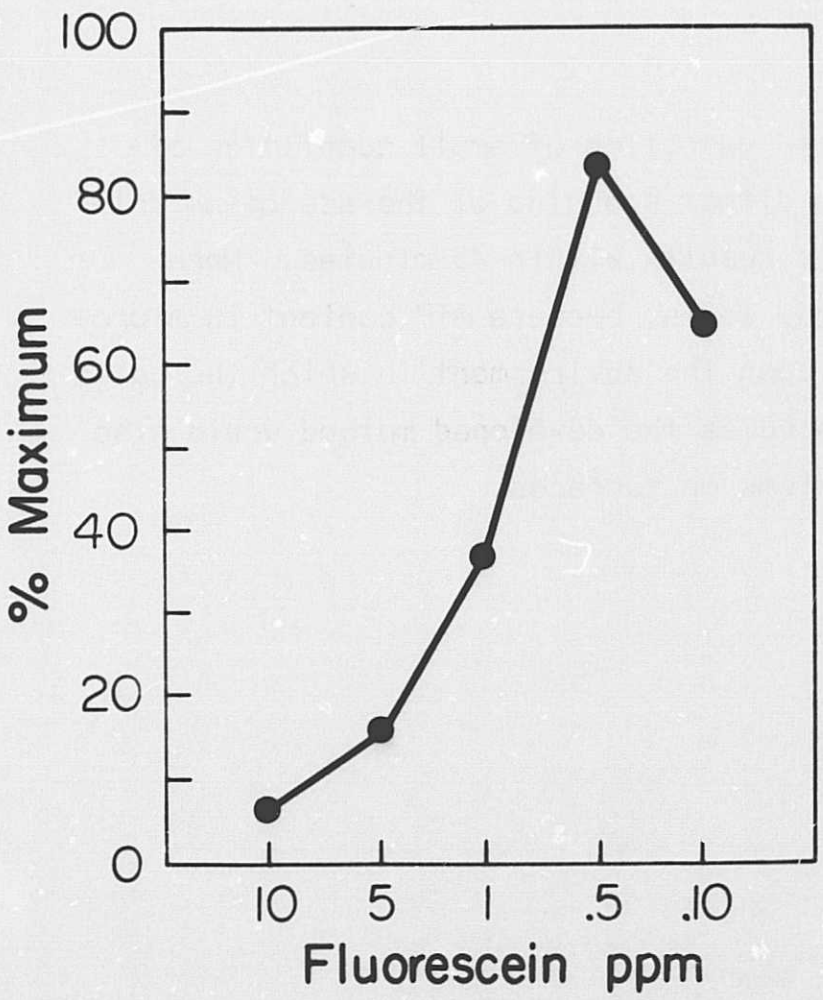

Figure 2.2 - Effect of fluorescein concentration on light emission from ATP.

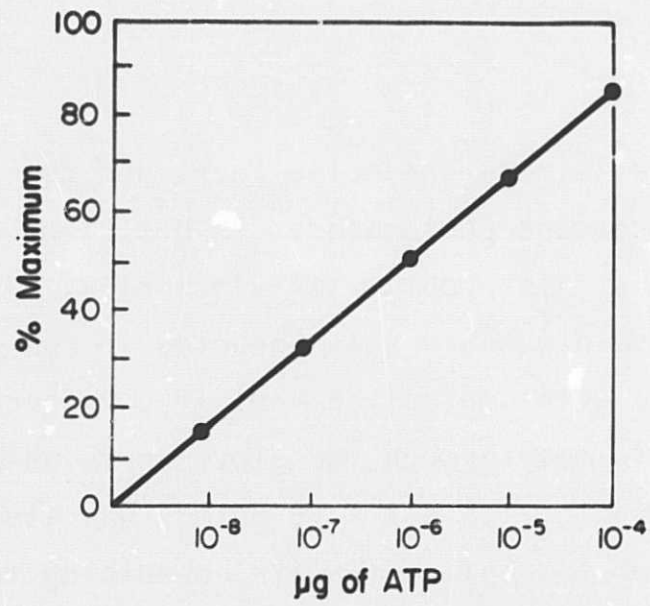

Figure 2.3 - Standard curve obtained with known amounts of ATP in solvent system containing 0.5 ppm of fluorescein

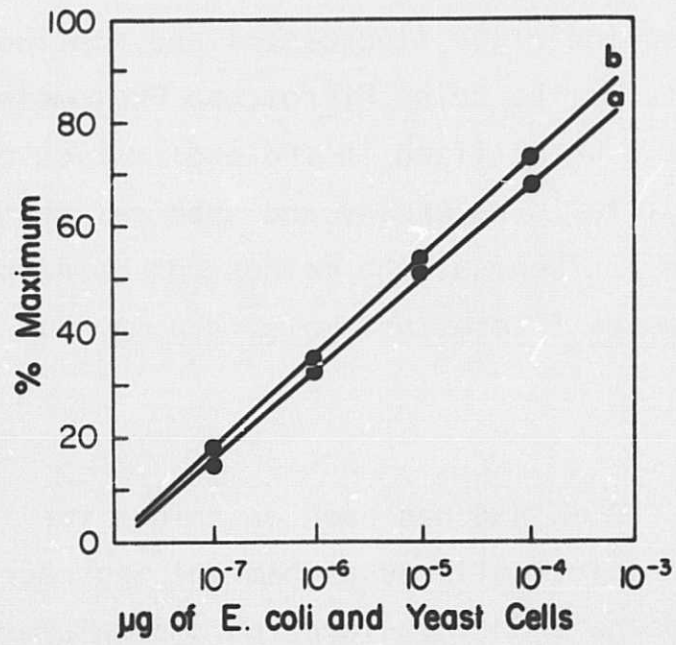

Figure 2.4 - A dilution curve of a suspension of E. coli (a) and yeast cells (b)

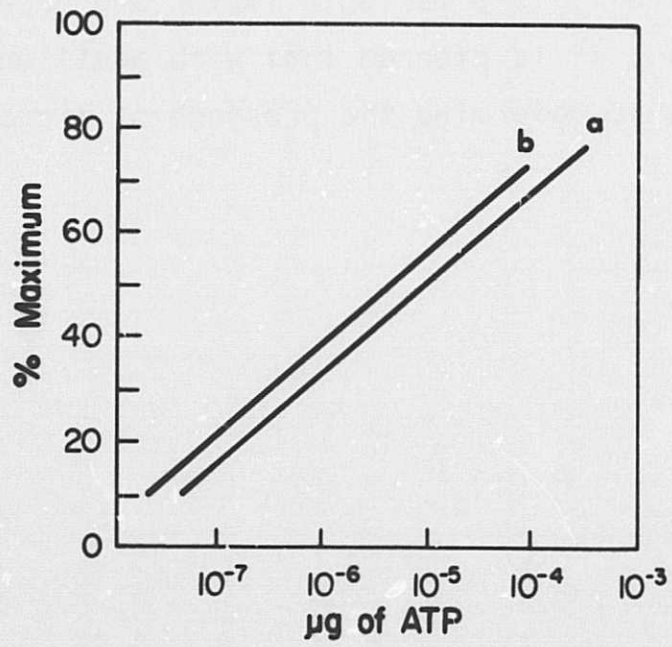

Figure 2.5 - Dilution curves of ATP (a) and ATP with 1×10^{-2} µg of E. coli (b)

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DRY HEAT DESTRUCTION RATES OF *BACILLUS SUBTILIS* VAR. *NIGER* IN A CLOSED SYSTEM

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Division of Environmental Health

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Division of Biometry

INTRODUCTION

This is a report of the continuation of efforts toward attaining the project objectives outlined in Progress Report #5, December, 1970. Experiments were carried out using the same basic cylindrical heat block system described in Progress Report 3, December, 1969.

OBJECTIVE

In review, the objective of this project is to determine the dry heat D-values of microorganisms in closed systems. Specifically it is to determine: 1) the relationship of spore D-value to spore water content, 2) the effect of atmospheric volume per spore on the D-value of spores with different initial water contents, 3) the effect of pressure on D-value, and 4) the effect of water adsorption and water vapor transfer characteristics of plastic materials on the D-values of spores encapsulated in plastic and spores on metal strips pressed against a plastic surface.

Results obtained from studies on the effects of water vapor pressure and relative humidity on the D_{125}-value of spores and the shape of the survivor curve are presented in this report.

EXPERIMENTAL PROCEDURE

In order to determine the effects of water vapor pressure and relative humidity on the survival pattern of spores using the heat block system the heat blocks were equilibrated and assembled in an atmosphere of known temperature and relative humidity.

The starting point in all tests was clean and dry heat blocks, gaskets and stainless steel discs. *Bacillus subtilis* var. *niger* spores were deposited on stainless steel discs in a clean room and air dried. These inoculated discs and the block components were allowed to equilibrate overnight in a plastic glove box isolator in which the relative humidity and temperature were controlled. After

equilibration the block units containing the inoculated discs were assembled and sealed while inside the glove box.

The relative humidity within the plastic isolators was maintained by adding a given volume of water to a given weight of silica gel which was placed in the bottom of the isolator (method described in Appendix A of Progress Report 3, December, 1969). The relative humidity was measured using a Honeywell Model W6111A electronic indicator with a range from 2-100% RH. Tests were carried out at room temperature controlled at 22°C and also at 7 and 37°C by placing the plastic isolator in temperature-controlled environmental rooms at these temperatures. The conditions evaluated are listed below.

Temperature °C	Relative humidity, %
7	35, 80
22	<2, 35, 80
37	35, 80

When the heat blocks for an individual experiment had been assembled and sealed, they were removed from the plastic glove box and subsequently heated in the oil bath at 125°C for varying lengths of time. Following heating they were removed from the oil bath and cooled in an ice water bath. The discs were then removed from the blocks and assayed for survivors by the plate count method.

A lag correction factor of 12.5 minutes was subtracted from each time interval to correct for the lag in the heating and cooling of the blocks. The times used in constructing survivor curves and calculating D-values are, therefore, equivalent times at 125°C.

All data were analyzed using a digital computer and appropriate program (see Appendix A). The output for each experiment included a D-value with 95% confidence limits, y-intercept, intercept ratio (IR), graph of the number of survivors versus the equivalent heating time at 125°C, and other pertinent statistical parameters. D-values were determined from the slope of the line of least-squares excluding the zero-time data (initial population N_o).

Studies have been initiated to measure the water in spores and adsorbed on stainless steel surfaces in the heat block systems using the same type water analyzer (CEC moisture analyzer 26-321A) employed by Dr. J.E. Campbell of Cincinnati in his NASA research studies. Work to date in this area has been concerned with planning the program and carrying out preliminary experiments to determine the type of system to be used and the experimental procedures that must be followed in order to develop these data.

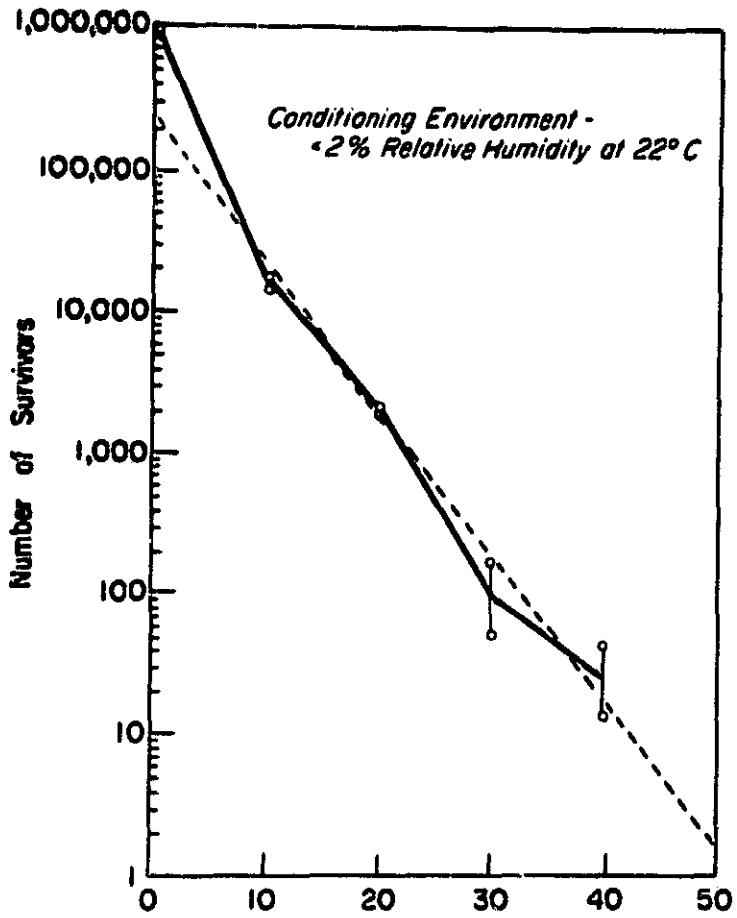

Figure 3.1 - Survivor curve for AAOE spores at 125°C - 2% RH, 22°C

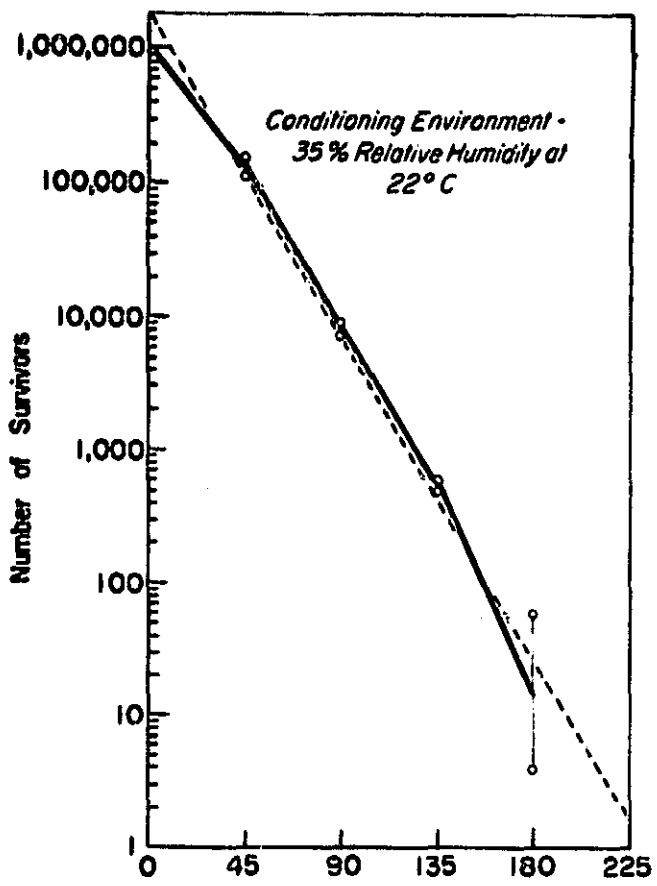

Figure 3.2 - Survivor curve for AAOE spores at 125°C - 35% RH, 22°C

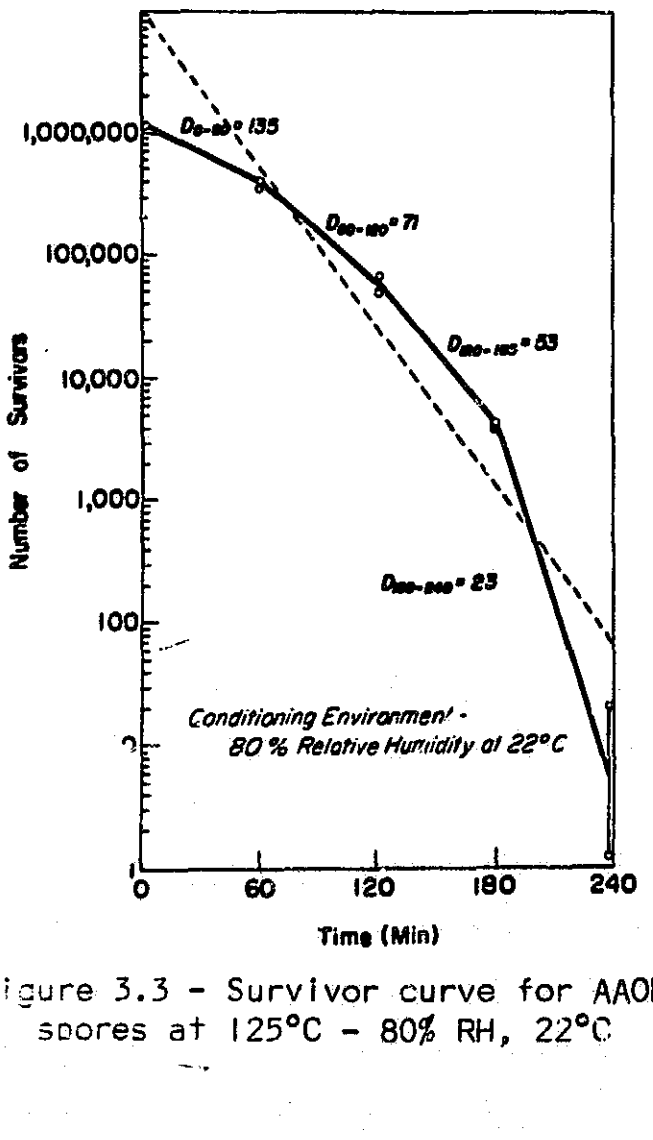

Figure 3.3 - Survivor curve for AAOE spores at 125°C - 80% RH, 22°C

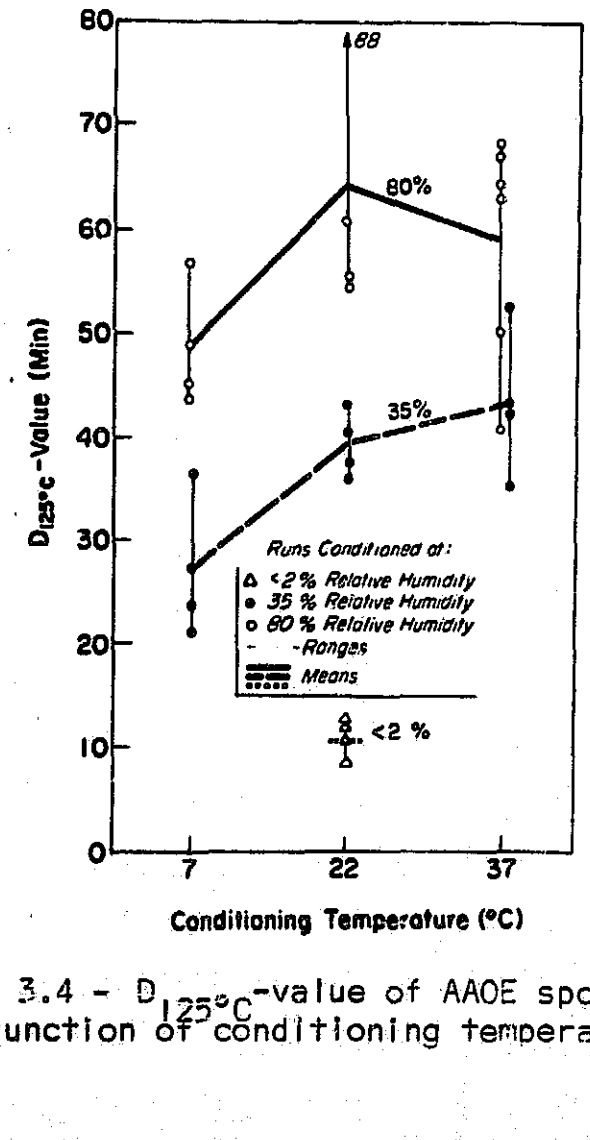

Figure 3.4 - $D_{125°C}$-value of AAOE spores as a function of conditioning temperature

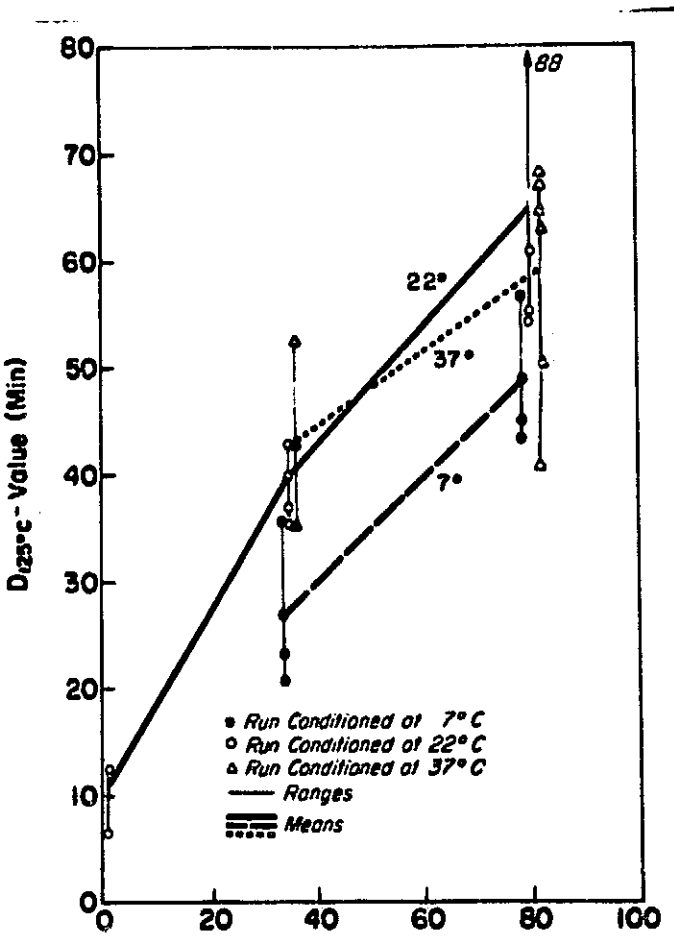

Figure 3.5 - D-value of AAOE spores as a function of the conditioning RH

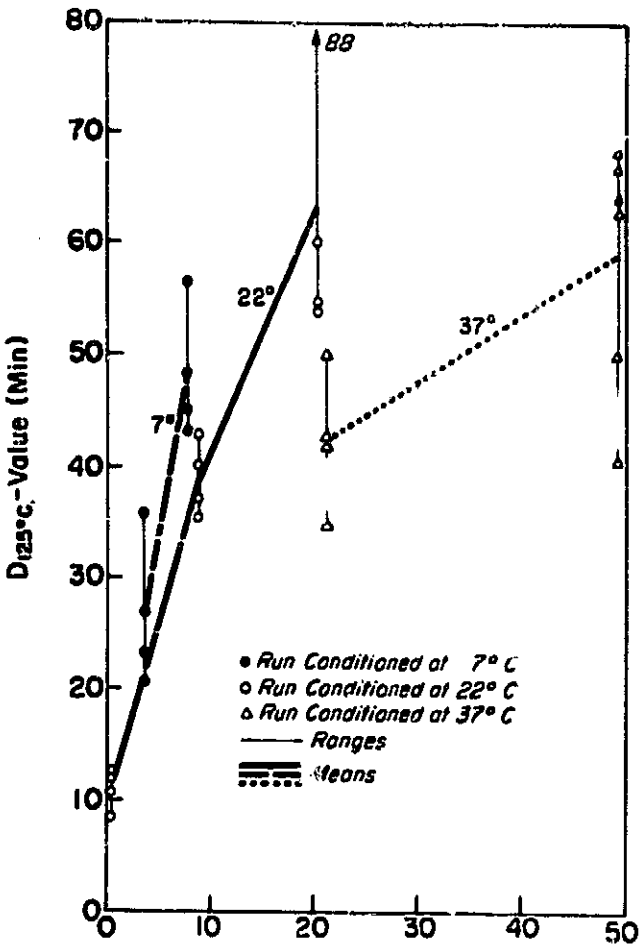

Figure 3.6 - $D_{125°C}$-value of AAOE spores as a function of water vapor pressure

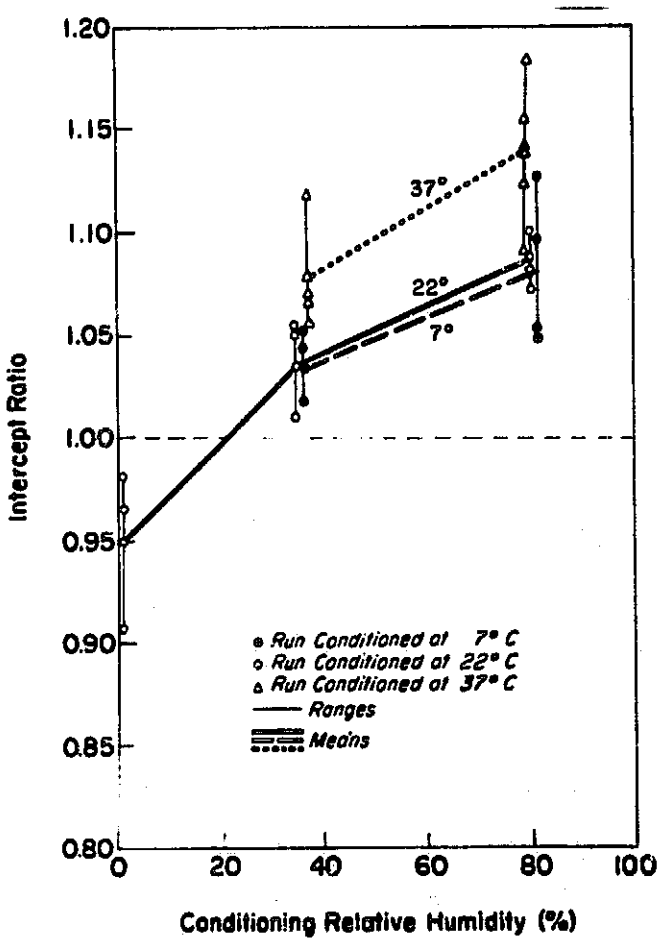

Figure 3.7 - Intercept ratio of survivor curves for AAOE spores at 125°C as a function of the conditioning RH

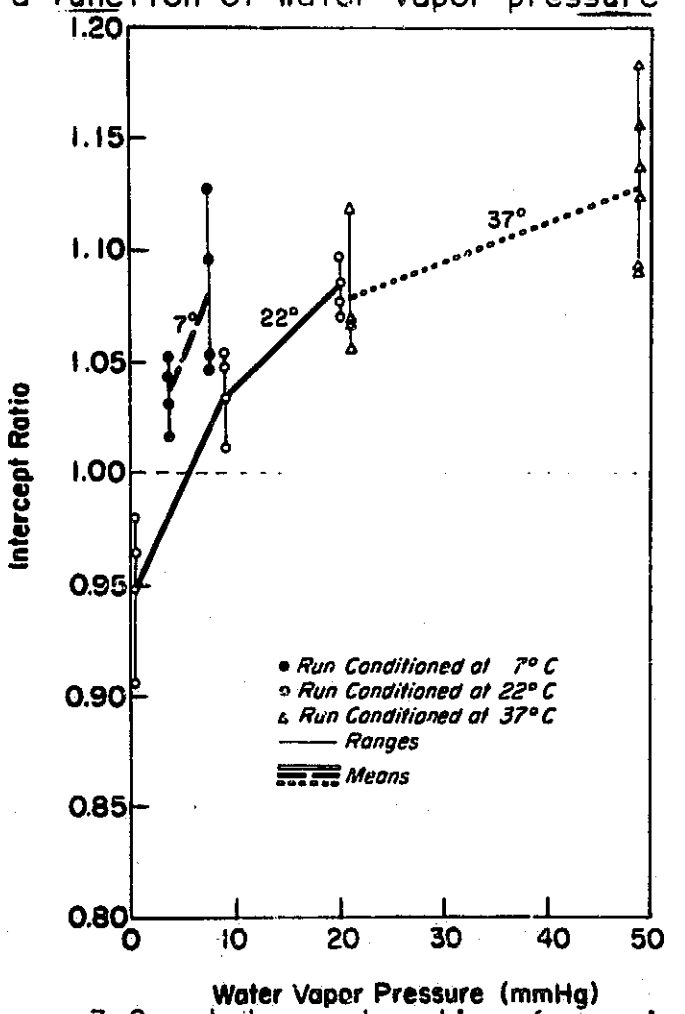

Figure 3.8 - Intercept ratio of survivor curves for AAOE spores at 125°C as a function of the water vapor pressure

RESULTS AND DISCUSSION

The survivor curves from different test conditions differ in shape. For spores conditioned at <2% RH the initial die-off rate was higher than the die-off rate during later time periods (Figure 3.1). When the initial die-off rate is higher, the y-intercept falls below N_o and the IR (log y-intercept ÷ log N_o) is less than one. For spores conditioned at 35% RH (Figure 3.2) the IR was greater than one. The survivor curve obtained for the 80% RH conditioning environment (shown in Figure 3.3) is in reality a curve, where as time increases the D-value decreases. We have calculated a D-value for each of the four segments of the survivor curve and these values are shown on the appropriate segments of the curve in Figure 3.3.

In Figure 3.4 we have plotted D_{125}-values as a function of temperature to indicate that these preliminary results show a different effect at 80% than at 35% RH. However, the question exists whether, in view of the shape of the 80% RH survivor curve, we can meaningfully calculate a D-value for survivor curves of this shape.

In Figures 3.5 and 3.6 we show the D_{125}-value results as functions of conditioning relative humidity (Figure 3.5) and water vapor pressure at the test temperature of 125°C (Figure 3.6). At all three temperatures (7, 22 and 37°C) the D_{125}-value increased as the conditioning relative humidity increased (Figure 3.5). When the D-value data are plotted versus relative humidity (Figure 3.5) the results at 22 and 37°C do not differ significantly from each other whereas the results at 7°C do appear to be different.

When the D-value data are plotted as a function of water vapor pressure at 125°C (figure 3.6) the resulting data at 37°C are different from the data at 7 and 22°C; the 7 and 22°C data also appear to be different from each other. A comparison of the data in Figures 3.5 and 3.6 suggests that results can be predicted more accurately on the basis of conditioning relative humidity than vapor pressure.

The intercept ratios for the several experiments have been plotted as a function of conditioning relative humidity in Figure 3.7 and as a function of the water vapor pressure at test temperature in Figure 3.8. The data in Figure 3.7 suggests that there is a general increase in the intercept ratio as the conditioning relative humidity increases. The intercept ratios at 35 and 80% RH were the same at 7 and 22°C, however, the IR at 37°C appeared to be higher for the spores conditioned at 35 and 80% RH. Considering all conditions tested, only the <2% RH condition (22°C0 had an IR of less than one. The 22°C results suggest that at relative humidities between 15 and 20% the IR will be one, at <15% RH the IR will be <1, and at >20% RH the IR will be >1.

In the graph of intercept ratio as a function of water vapor pressure (Figure 3.8) the IR increased as the vapor pressure increased for all three temperatures evaluated. In general it does not appear that there is a relationship between IR and vapor pressure at one conditioning temperature compared to another conditioning temperature.

In the experimental system used to gather these data the temperature and relative humidity in the glove box isolator were controlled. While located inside the isolator the spore discs and the blocks were equilibrated and then assembled into sealed heat block units. Assuming zero leakage and/or no diffusion through the Teflon gasket, the quantity of water in the cavity inside the heat blocks during the heat treatment period will be the same as it was during sealing. It is this initial total water content that has been varied among experiments. The water condition of the gas inside the cavity at the time the heat blocks were sealed can be described by the temperature and relative humidity of this gas or, since the glove box isolator is in equilibrium, by the temperature and relative humidity inside the isolator. As far as vapor pressure and relative humidity are concerned the water condition inside the block will change as the heat block temperature changes from the ambient sealing temperature to the heating temperature. If we know the volume of the heat block cavity and assume that all the water present in the cavity at the time of sealing is in the gaseous phase, we can calculate the total pressure, water vapor pressure, and relative humidity inside the cavity at the heating temperature.

Due to the adsorption of water on all surfaces and the presence of water inside the microbial spores, the water in the gaseous phase in the cavity will not be the only water present. It is imperative that we have data regarding the quantity of water adsorbed on surfaces inside the heat block cavity. These studies are underway but present a severe challenge tue to the small quantities of water that must be quantitatively measured.

CONCLUSIONS

1. D-value increases as conditioning relative humidity (<2-80%) increases and in general as vapor pressure increases.

2. The rate of initial die-off decreases with increasing relative humidity and vapor pressure.

3. A straight line logarithmic survivor curve appears to be an inappropriate description of the survivor curves for spores in systems conditioned at 80% RH.

FUTURE WORK

1. Studies on the effect of relative humidity - water vapor pressure will be

continued until a relationship of microbial destruction with either relative humidity or water vapor pressure has been clearly established.

2. The sealing efficiency of the heat-block system will be reevaluated.

3. Measurement will be made of the water in the spores on stainless steel discs and adsorbed onto stainless steel surfaces when subjected to different relative humidities.

DRY HEAT DESTRUCTION RATES OF MICROORGANISMS ON SURFACES

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Division of Environmental Health

Project Contributors: R. Jacobson
Division of Biometry

INTRODUCTION

In previous studies of the dry heat destruction rates of microorganisms on surfaces, spores of Bacillus subtilis var. niger have been used as the test organism. The recent concern of the NASA Planetary Quarantine Office regarding the probability that small particles of soil with their accompanying microflora may accumulate on the lander during assembly and influence sterilization requirements has caused us to direct our attention toward determining whether or not soil particle size has a role in the dry heat resistance pattern of spores in soil.

Review of literature

Koesterer (1963) reported that when 0.1 gm of garden soil containing more than 10^6 microorganisms was subjected to a dry heat sterilization cycle more than ten microorganisms survived after sixty hours at 125°C. He was able to obtain a $D_{125°C}$-value of about ten hours for the laboratory grown spores of isolate 541. This organism was isolated from soil. Favero and co-workers (1970) reported survival of microorganisms in a Cape Kennedy soil sample after long heating times. When subjected to dry heat the survival characteristics of the spores in the Cape Kennedy soil were of the same order of magnitude as the spores in garden soil.

Doyle and Ernst (1967) subjected Bacillus subtilis var. niger spores occluded in crystals of calcium carbonate to wet and dry heat and ethylene oxide. The resistance of the spores in crystals to inactivation by wet heat was 900 times greater and to dry heat 9 times greater than the resistance of non-occluded organisms. In general, spores occluded in crystals and subjected to ethylene oxide sterilization were not killed at all. Mullican and Hoffman (1968) reported that when spores of Bacillus subtilis var. niger were inside water soluble crystals of sodium chloride, the resistance of these spores to death by dry heat was six times greater than for unprotected spores.

It is an accepted fact that the quantity of water associated with spores during a dry heat treatment has an effect on spore survival. Increasing the quantity of water in the dry system, while maintaining the relative humidity at or below about 20%, increases the dry heat resistance of the microbial spores. Soil dried at room

temperature still contains a large quantity of adsorbed water and water of crystallization. During dry heat sterilization, water will be desorbed and some water of crystallization will be lost. Microbial spores in crystals or adjacent to soil particles may be protected by this water.

Conclusions that can be drawn from these data are:

1. Microorganisms in soil have an unusually high resistance to death by dry heat.

2. The resistance of microbial spores occluded in crystals to death by dry heat is increased six to nine times compared to non-occluded spores.

3. Microbial spores grown in the laboratory from spores that have survived long heating times in soil are themselves very resistant to dry heat.

4. Dry soil contains adsorbed water and water of crystallization.

5. The high resistance of microbial spores in soil to death by dry heat may be due to: (a) the natural resistance of the spores to dry heat, (b) the occluding of spores in crystals in the soil which protects the spores against water loss (c) the protective activity of the soil itself in supplying water to the micro-environment of the spore during exposure to dry heat or (d) more probably to a combination of all these conditions.

OBJECTIVE

The objective of these studies is to develop a better understanding of the resistance of microorganisms in soil to destruction by dry heat. We are specifically attempting to determine if there is a relationship between the size of the soil particles and the dry heat resistance of the spores attached to or occluded by these particles.

EXPERIMENTS PERFORMED AND RESULTS OBTAINED

We have carried out a series of preliminary experiments. The procedures followed and results obtained from these experiments are reported below.

Comparison of the survival of microorganisms in a Cape Kennedy soil with the survival of microorganisms in a Minnesota soil

A surface sample of soil was obtained from the Dinkytown area of Minneapolis, Minnesota adjacent to the Burlington Northern railroad right-of-way. The soil was transported to our laboratories and dried. The dry soil was sieved using a procedure similar to that described by Favero *et al.* (1970) except that we eliminated the ethanol washing, insonation, overnight settling and membrane filtration steps. We sieved the soil in a dry condition. The final sieve size was 43μ. After we had

obtained the dry particles that were smaller than 43 microns we weighed out a quantity of this soil and added ethanol at the ratio of 2 gm soil per 60 ml ethanol (same ratio used by Favero). As a final step the samples were titered to determine the number of colony-forming units per ml of the soil-ethanol suspension.

Tests were conducted to establish a survivor curve for both the Dinkytown soil-ethanol suspension and the Cape Kennedy soil-ethanol suspension (obtained from Favero). The planchet-boat-hot plate system was utilized in these experiments. The soil-ethanol suspension was maintained through the use of a magnetic stirrer system; .01 ml of the suspension was pipetted onto the planchets, the planchets were allowed to dry and equilibrate in the clean room (50 % RH, 22°C) for about 18 hours after which time heating was carried out by placing the planchets on boats which in turn were placed on the hot plate for heating times of 0.5, 1, 2, 4 and 6 hours. (The hot plate was located in the clean room.) The results of this experiment are shown in the following table.

Table 4.1

Comparison of the Number of Colony-Forming Units Surviving in Cape Kennedy Soil and Dinkytown Soil at 125°C

Heating Time(hrs)	Mean Number of Survivors per Planchet	
	Dinkytown soil	Cape Kennedy soil
0	683	300
0.5	85	62
1.0	30	30
2.0	19	29
4.0	9	16
6.0	2	6

The results above suggest that there are similarities in the survival of the microflora in the Cape Kennedy and the Dinkytown soil.

Heat Activation

Experiments were carried out to determine whether the initial numbers of colony-forming units could be increased by subjecting the soil-ethanol suspension to heat activation at 90°C for ten or thirty minutes.

In this experiment, one vial each of the Cape Kennedy soil and the Dinkytown soil was used. The vials contained a soil-ethanol suspension of particle size less than 43 microns.

The experiment was designed to measure the change in the plate count (number of colony-forming units) as the result of heat activation. The procedure of

inoculating and then recovering the spores from planchets normally followed in measuring dry heat destruction rates using the hot plate-boat-planchet system was used.

Three planchets were inoculated with 0.01 ml of each soil suspension to provide the initial number (no heat activation values).

The soil suspension remaining in each vial was divided into two portions. The test tube containing one portion of the soil suspension was heated for ten minutes and the test tube containing the other portion heated for thirty minutes at 90°C in a water bath. At the end of the heating period the tubes were cooled in an ice water bath. Three planchets were then inoculated with 0.01 ml of the soil suspension from each tube for each heat activation treatment (four treatments, three planchets per treatment).

The inoculum on the planchets was allowed to dry in the clean room. After the inoculum was dry, the planchets were transferred to a flask, 50 ml of buffered distilled water were added, the planchets were insonated for two minutes, after which a sample of the eluate was plated in duplicate using trypticase soy agar. The results are shown below.

Table 4.2

Results of Heat Activation at 90°C
for 10 min. and 30 min.

Heating Time(min)	Mean Number of Colonies per Planchet	
	Cape Kennedy soil	Dinkytown soil
0	4.10×10^3	3.73×10^4
10	3.30×10^3	3.34×10^4
30	2.12×10^3	2.36×10^4

Effect of particle size on the dry heat destruction rate of microbial spores associated with these soil particles

An experimental program was developed to answer the question: "Does the size of the soil particles have an effect on the survival of microbial spores associated with these particles?" The general plan of attack was to separate the soil into fractions that contain soil particles of similar size then assay these separate fractions to determine the relative dry heat destruction rate of the microorganisms associated with these soil particles.

A review of the literature on methods for particle size analysis indicated that sedimentation methods could be used to separate particles over the size range 1-200 microns. We proceeded to separate both the Dinkytown and the Cape Kennedy soil into

fractions using sedimentation techniques. Since the suspensions were in ethanol, we proceeded to carry out all separations in ethanol.

We initially prepared a suspension containing particles smaller than 15 microns and compared the survival of the microorganisms in this suspension with the survival of microorganisms in the original suspension. From this point we proceeded to develop through a single separation technique suspensions calculated to contain particles <5 microns, >5 <10 microns, and >30 <43 microns. At a later time the single separation procedure was replaced by a multiple separation technique and <2.5 micron particles were separated. The sedimentation procedures used in these experiments are described in Appendix D.

In dealing with soil particles we must contend with a wide range of particle densities and a wide range of particle shapes. Since the sedimentation rate is calculated for spheres, and since the shape and density of the particles will affect their sedimentation rate the final suspensions we obtained can only be considered to contain particles that have sedimentation rates equivalent to spheres for the sizes given and for the density used in the calculations. We recognize that this method of separation is not ideal. We are looking for other methods to separate soil.

Four series of experiments were carried out in this study:

1. A comparison of the survival characteristics of the microbial spores associated with soil particles <15 microns and those of the original soil suspension containing particles <43 microns.

2. A comparison of the survival characteristics of the microbial spores associated with soil particles that were <5, >5 <10, and >30 <43 microns that were separated using a single separation technique.

3. A comparison of the survival characteristics of the microbial spores associated with soil particles that were <5, >5 <10, >30 <43 microns prepared using multiple separations.

4. A comparison of the survival characteristics of the microbial spores associated with soil particles <2.5, >2.5 <5, >5 <10, and >30 <43 microns obtained through multiple separations.

The results of experiments 2, 3, and 4 showed a single trend. Those microorganisms associated with the smaller sized particles died off more quickly than those associated with the larger sized particles. In experiments 3 and 4 the differences in surviving numbers or survival time as a function of particle size appeared to be sharper than in experiment 2. The results of experiment 1 were inconclusive; this result is not inconsistent since the relative differences in the particle size of the soil suspensions compared were small.

It was the plan to carry out both survivor curve and end point tests. The

survivor curves from both the Dinkytown and the Cape Kennedy soil are non-linear when the log of the surviving numbers of organisms is plotted as a function of heating time. Therefore, the relative shape of the survivor curve is important in observing the rate of change of the number of spores associated with the different particle size fractions. The end point data are critical as far as determining sterilization times. In general the survivor curve and end point experiments agreed in that in the survivor curve tests, as particle size increased the numbers of surviving organisms for a given time of heating increased and in the end point tests a longer heating time was required to reach the point at which none of the replicates showed growth.

In experiment 4 a test was conducted in which dry heat testing was carried out simultaneously both in the dry box where the quantity of water in the air was approximately 55 ppm (0.2% RH at 22°C) and in the clean room where the humidity was 50% at 22°C. The results of this single test showed no distinct difference in the microbial survival of spores associated with similar size particles between the two different environments. This result is quite different from the results that had previously been reported for Bacillus subtilis var. niger spores where the D-value was two to three times greater in the clean room than in the dry box.

Experiments 2, 3, and 4 were designed to determine whether after a specific dry heat treatment there are differences in the numbers of surviving organisms for the different particle size suspensions being evaluated. We decided that we would make our evaluation using soil suspensions with equal numbers of colony-forming units. We have made no attempt to establish a relationship between soil particle count and colony forming unit (viable particle) count. This relationship would be a function of the original soil sample. We are attempting to quantify the number of soil particles in the test suspensions by direct microscopic counting.

The different sized soil-particle suspensions were standardized so that for any single experiment the initial viable counts were the same for all samples evaluated. This was done by titering each soil suspension sample, diluting the concentrated samples to the level of the least concentrated sample and then re-titering all samples. If necessary, a second adjustment in viable count concentration was carried out followed by re-titering.

SUMMARY AND CONCLUSIONS

The studies that we have carried out in attempting to determine if there is an effect of particle size on the survival of microorganisms associated with these particles have consistently suggested that there is a relationship and that as the particle size decreases the survival time decreases. We believe that we have sufficient preliminary evidence to now proceed to carry out more elaborate separations so that we can

establish statistically the relationship of particle size and the survival times of the microorganisms associated with the different sizes of particles.

FUTURE WORK

We plan to continue the work directed toward determining whether there is a relationship between the size of soil particles and the microbial spores associated with these particles. We plan to proceed by carrying out larger separations which will give us large enough samples to run replicated tests to allow a statistical analysis to be made. Then we can determine if there is a relationship between size of particle and the microbial spores associated with it and to quantify this relationship.

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APPENDIX A: DATA HANDLING SYSTEM

The data from microbiological experiments conducted in our laboratories are recorded and stored on punched cards. Once the data have been placed on punched cards and verified, the cards containing the data can be used to prepare statistical analyses and graphs through the use of computer facilities. Subsequent analyses and interpretation of the experimental results then can be based on an examination of the computer output.

Our data handling system begins with the transfer of data from the original tabulation sheets to coding sheets and is concluded when the experimenter accepts the computer output. A flow chart of our data handling system is shown in Figure 1. A detailed description of the data handling system follows.

The majority of our original data is in the form of microbial colony counts resulting from our dry heat destruction rate experiments. Two or three dilutions are prepared from each test sample and two subsamples are plated from each dilution. The data collector records all of this information on the original tabulation sheet similar to the one shown in Table 1. The original data consists of colony counts for each dilution plated. In one sense the data for the two or three dilutions can be considered duplicate sets of data, however, only the data from one dilution is usable in terms of the guidelines that appear in Appendix E of Progress Report #3 (December, 1969). The one set of original data that is selected according to the guidelines is utilized in all subsequent analyses. These data are transferred to code sheets along with pertinent information about the experiment. A completed code sheet is shown in Table 2.

We have found that it is helpful to keep a record of the status of the data set in the data handling system. Therefore, when a code sheet has been completed and submitted for the data to be punched on cards the experiment number and date are recorded into a log book. The data are then punched on cards.

The computer program and data deck are submitted to the computer which calculates the statistical parameters that we have deemed necessary for our data. The computer output consists of a raw data table and several tables of summary statistics. The first part of the computer output, as shown in Table 3, consists of the raw data table and is verified with the original tabulation sheets. If no errors are found in the raw data table all tables in the computer printout are accepted for further study.

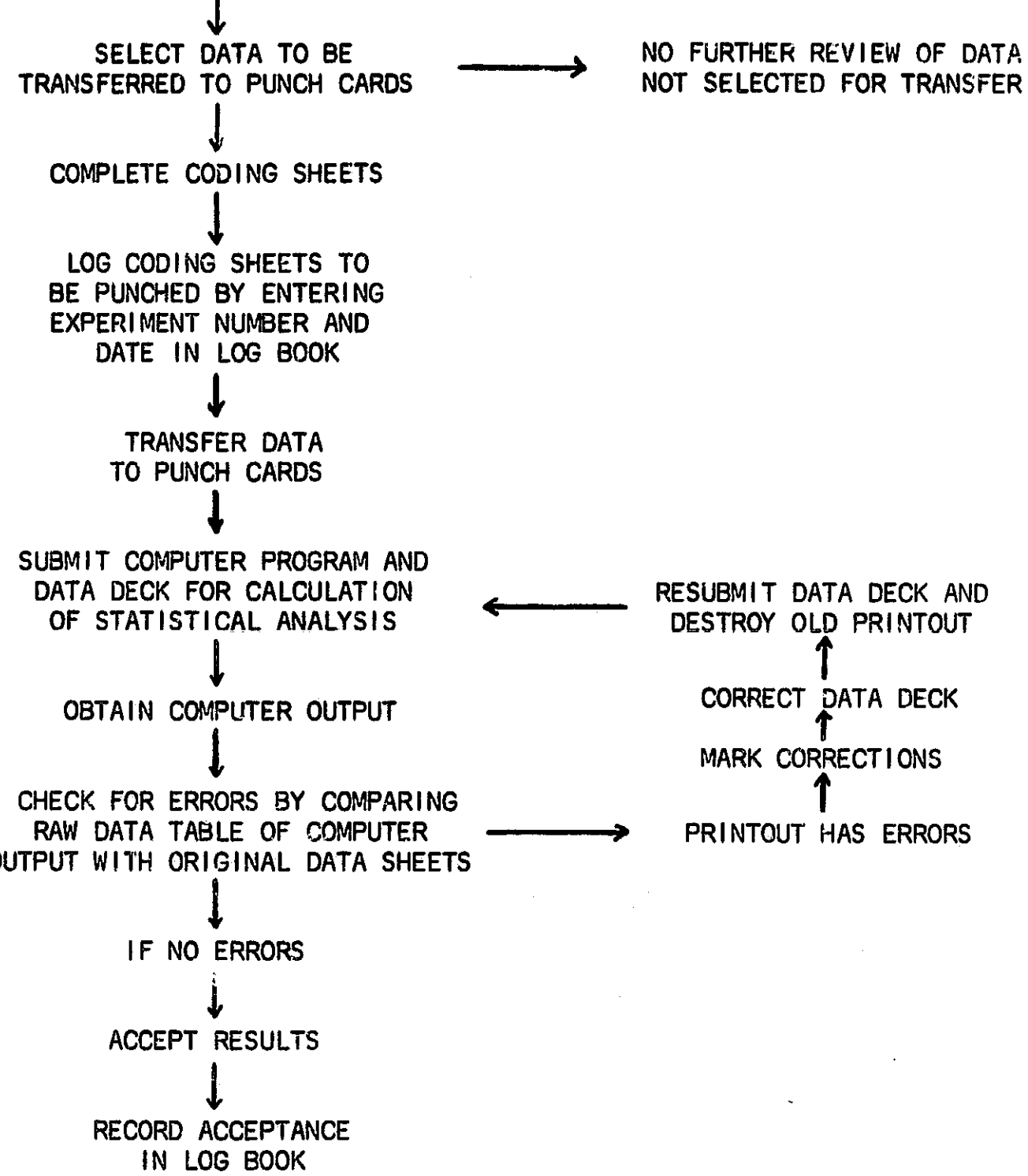


Figure 1 - Flow chart of data handling system

Table 1

Raw Data Tabulation Sheet

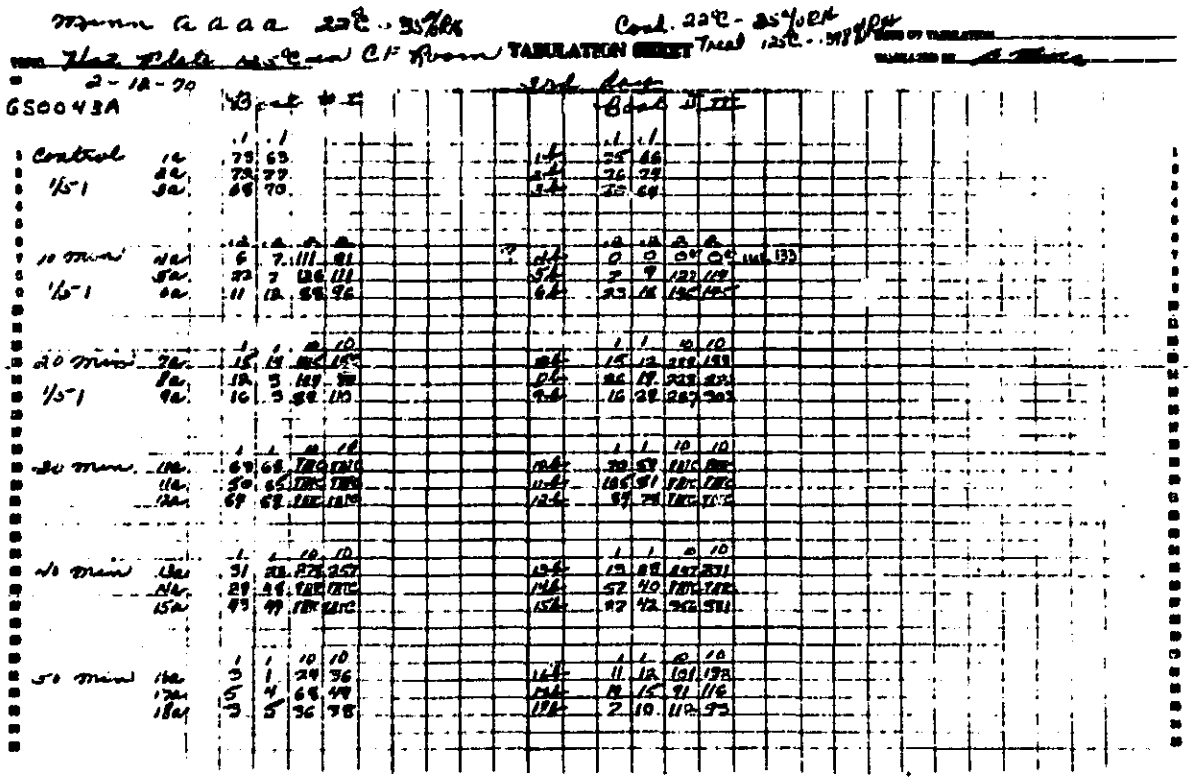


Table 2

A Filled-in Code Sheet

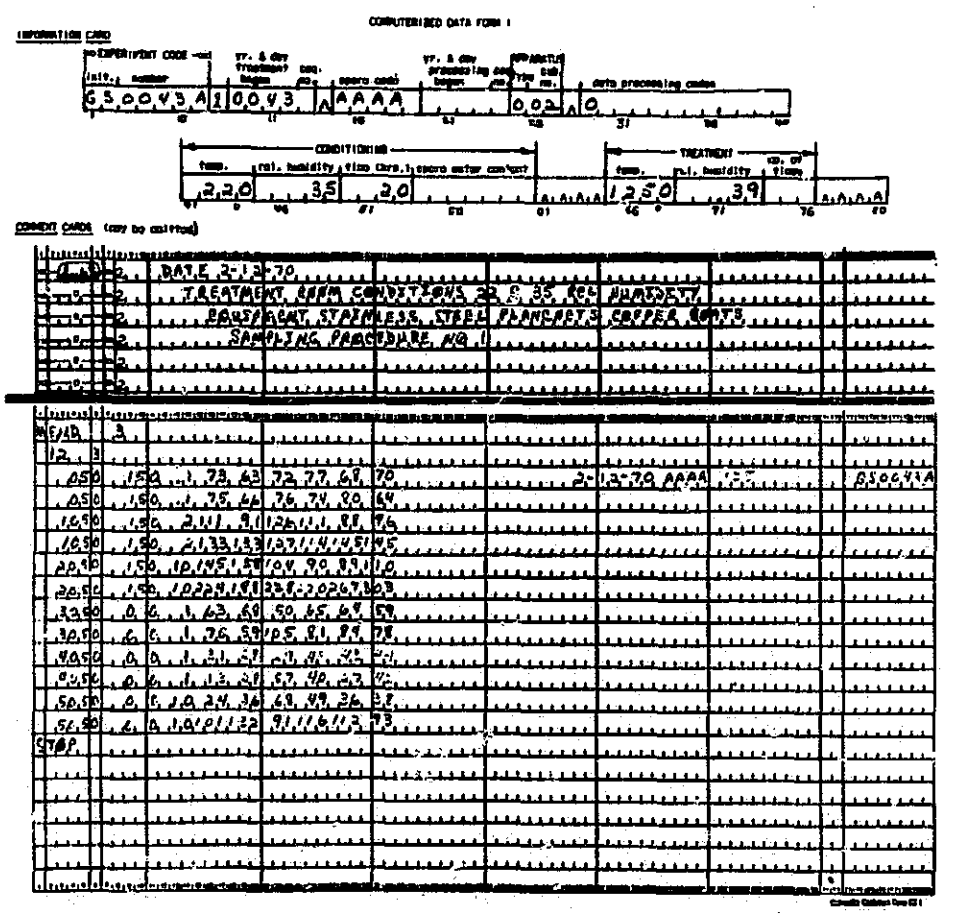


Table 3

Raw Data Table from Computer Output

DATA TABLE FOR EXPERIMENT NUMBER GS0043A
EXPERIMENT STARTED ON DAY-043, YEAR-1970, SPORES-AAAA.
APPARATUS TYPE OPEN, NAME-HOT PLATE IN CLEAN ROOM
SAMPLES CONDITIONED AT 22.0 C, 35 PCT. R.H. FOR 20 HOURS
SAMPLES TREATED AT 125.00 C, .39 PCT. R.H. FOR TIMES LISTED.
COMMENTS - DATE 2-12-70
TREATMENT ROOM CONDITIONS 22 C 35 REL HUMIDITY
EQUIPMENT STAINLESS STEEL PLANCHETS COPPER BOATS
SAMPLING PROCEDURE NO 1

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	0	50.00	1.00	51.00	.10	25500.0000	3	2	1794275.43	6.253889	73	63	72	77	68	70
2	0	50.00	1.00	51.00	.10	25500.0000	3	2	1843006.75	6.265527	75	66	76	74	80	64
3	10.00	50.00	1.00	51.00	2.00	1275.0000	3	2	131314.34	5.118312	111	91	126	111	88	96
4	10.00	50.00	1.00	51.00	2.00	1275.0000	3	2	168798.34	5.227368	133	133	127	114	145	145
5	20.00	50.00	1.00	51.00	10.00	255.0000	3	2	28854.98	4.460221	145	158	104	90	89	110
6	20.00	50.00	1.00	51.00	10.00	255.0000	3	2	60070.29	4.778660	224	188	228	220	267	303
7	30.00	50.00	0	0	1.00	50.0000	3	2	3060.30	3.485764	63	68	50	65	64	59
8	30.00	50.00	0	0	1.00	50.0000	3	2	3914.14	3.592636	70	59	105	81	84	78
9	40.00	50.00	0	0	1.00	50.0000	3	2	1814.75	3.258817	31	28	29	48	43	44
10	40.00	50.00	0	0	1.00	50.0000	3	2	1565.23	3.19	78	13	28	57	40	27	42
11	50.00	50.00	0	0	10.00	5.0000	3	2	198.70	2.298187	24	36	68	49	36	38
12	50.00	50.00	0	0	10.00	5.0000	3	2	532.91	2.726653	101	132	91	116	112	93

COLUMN TITLES

(1) = IDENTIFICATION
(2) = HEATING PERIOD
(3) = VOLUME(ML) ORIGINAL SUSPENSION (INCLUDE SAMPLE)
(4) = AMOUNT TRANSFERRED (ML)
(5) = VOLUME (ML) IN RECEPTACLE AFTER TRANSFER
(6) = AMOUNT (ML) PLATED FROM (5) (FROM (3) IF (5) = 0)
(7) = DILUTION FACTOR
(8) = NUMBER OF SAMPLES PER EXPERIMENTAL UNIT
(9) = NUMBER OF PLATES PER SAMPLE
(10) = ESTIMATED NUMBER OF SURVIVORS IN ORIGINAL SUSPENSION
(11) = LOG10 OF (10)
(12) = PLATE COUNT 1 FOR SAMPLE 1
(13) = PLATE COUNT 2 FOR SAMPLE 1
(14) = PLATE COUNT 1 FOR SAMPLE 2
(15) = PLATE COUNT 2 FOR SAMPLE 2
(16) = PLATE COUNT 1 FOR SAMPLE 3
(17) = PLATE COUNT 2 FOR SAMPLE 3

When the computer printout has been returned to the experimenter and when he has accepted it as being correct this fact is noted in the log book.

If errors are detected in the raw data table of the computer printout, the raw data table becomes a coding sheet and corrections are marked on it. The data cards are corrected and the original printout is destroyed. The corrected data deck is then rerun and a new computer printout obtained. This is examined in the same way as the first printout. The same path is followed until all known errors are corrected and the final computer printout is accepted for further study.

The computer printout consists of several tables of summary statistics. The description statistics for each experimental condition are shown in Table 4. In Figure 2 is shown the computer prepared graph of results. The computer draws a point-to-point line through the mean value points and then indicates the data point for each experimental unit and the confidence interval for each experimental condition. D-values calculated from adjacent experimental conditions are given in Table 5. The regression statistics for the experiment are shown in Table 6. The results of the analysis of variance and the calculated intercept ratio are shown in Table 7.

Data sheets and cards are stored for future use. The original data sheets are placed in three-ring notebooks; the computer outputs are assembled into binders. The data cards are stored in a centrally located card cabinet.

This system of handling and storing data makes possible a rapid statistical analysis of the data with minimum labor input by the experimenter. The experimenter is provided with a convenient record to use in evaluation of the data. The system includes checks for validating the correctness of the data that enters the system. Our system also provides an organized method for retaining data from our experiments. An added advantage of this method of handling the data is that since the data are on punched cards, additional analyses can be performed at a later date with a minimum amount of effort and chance for error. The preparation of data tables can be made from special printouts which will minimize the labor requirements and errors.

Table 4

Descriptive Statistics for Each Experimental Unit

X(I)

0	6.2539	6.2655
10.0000	5.1183	5.2274
20.0000	4.4602	4.7787
30.0000	3.4858	3.5926
40.0000	3.2588	3.1946
50.0000	2.2982	2.7267

I NO	X-VALUE	Y-MEANS	VAR(Y/X)	S.D.(Y-MEAN)	U-C.I.	L-C.I.
1 2	0	6.259708	.000068	.005819	6.333651	6.185765
2 2	10.000000	5.172840	.005947	.054528	5.865743	4.479938
3 2	20.000000	4.619440	.050702	.159219	6.642687	2.596194
4 2	30.000000	3.539200	.005711	.053436	4.218228	2.860173
5 2	40.000000	3.226697	.002063	.032119	3.634846	2.818549
6 2	50.000000	2.512420	.091791	.214233	5.234733	-0.209893

POOLED SS+ .15628 DF= 6

POOLED VARIANCE= .02605

SD-POOLED= .16139

Table 5

D-values Calculated from
Adjacent Time Periods

TIME	AVERAGE LOG	N	ANTILOG	D-VALUE
	6.2597	2	1818477.86	
5.00				9.20
10.00	5.1728	2	148881.30	
15.00				18.07
20.00	4.6194	2	41633.24	
25.00				9.26
30.00	3.5392	2	3460.99	
35.00				32.00
40.00	3.2267	2	1685.38	
45.00				14.00
50.00	2.5124	2	325.40	

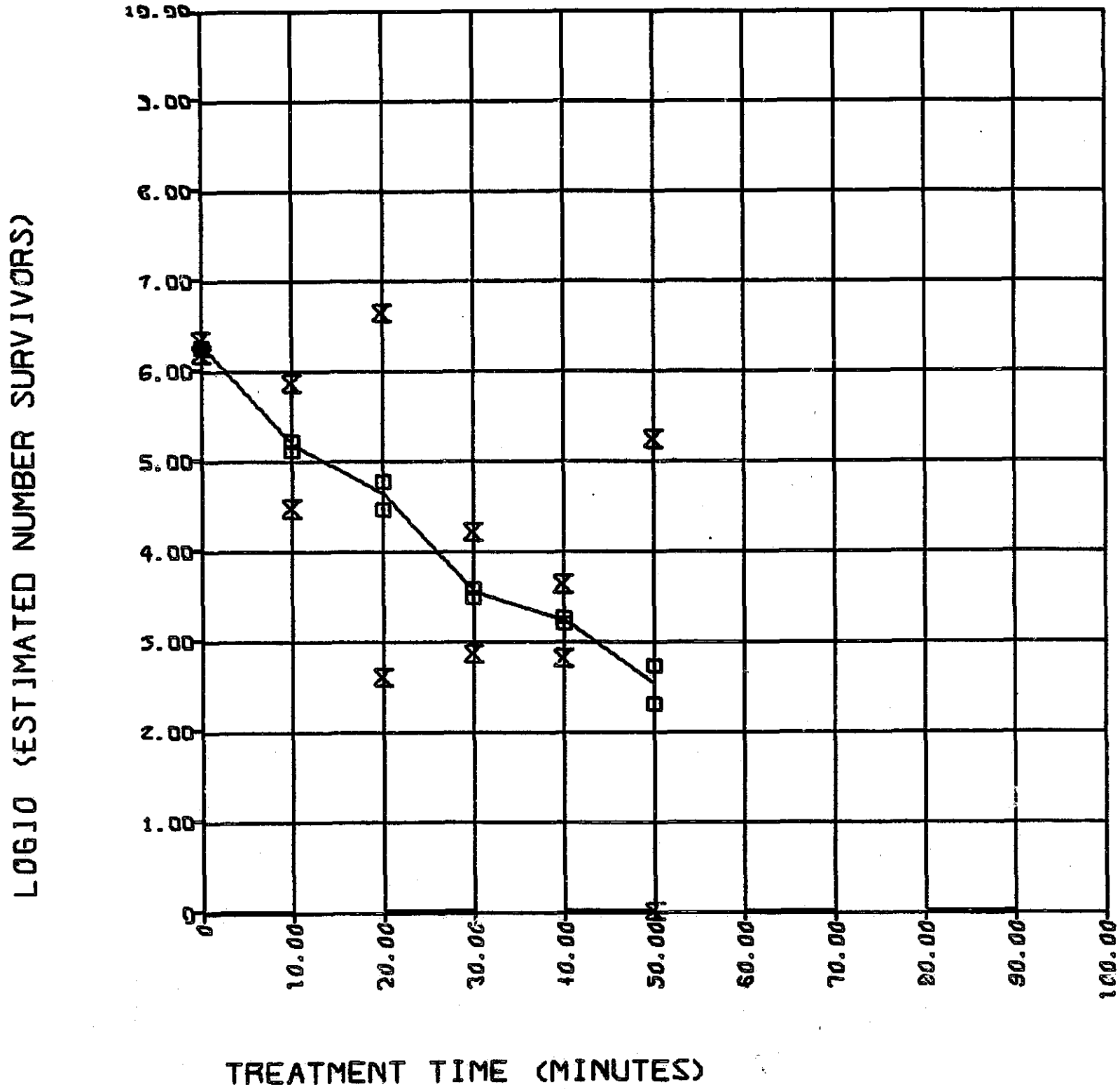


Figure 2 - Computer prepared graph of experimental results: The line passes through sample means. Both data points (⊡) and confidence limits (⧖) are shown.

Table 5

Regression Statistics for the Experiment

INFORMATION ABOUT Y AT X = 0

MEAN = 6.2597080183

VARIANCE = .0000677202

95 PER CENT CI= 6.1857650880 , 6.3336509486

ANTILOG MEAN = 1818477.76

ANTILOG 95 PER CENT CI = 1533787.13 2156010.88

THE FOLLOWING CALCULATIONS EXCLUDE INFORMATION ABOUT Y OP X AT X = 0

SUMMARY STATISTICS

D-VALUE = 14.8952 95 PER CENT CI = 12.8044 , 17.8020

SLOPE = -0.0671358303 95 PER CENT CI = -0.0781 , -0.0562

INTERCEPT = 5.8282 95 PER CENT CI = 5.4746 , 6.1918

SLOPE/SQRTK = -3.0024056041

ANTILOG INTERCEPT = 673278.18

ANTILOG 95 PER CENT CI = 291482.72 1555164.29

Table 7

Analysis of Variance Table

ANOV

SOURCE	DF	SS	MS	F	P
MEAN	1	145.4750858359	145.4750858359	4656.2875	.0000
TRT	4	9.2194601223	2.30486 0306	73.7729	.0001
REG	1	9.0144394115	9.0144394115	288.5293	.0000
DEV	3	.2050207108	.0683402369	2.1874	.2079
ERR	5	.1562136002	.0312427200		
TOT	10	154.8507595584			

INTERCEPT RATIO = .9310649195

APPENDIX B: A REVIEW OF NATURALLY OCCURRING INTERIOR MICROBIAL CONTAMINATION OF SPACE HARDWARE

Introduction

Efforts to evaluate the microbial contamination potential of "shelf-item" space hardware have been hampered by a lack of proven recovery techniques suitable to this task and also by difficulty in measuring and controlling background contamination levels which make positive results uncertain. On the other hand, toxicity of the material and possible bactericidal effects of grinding, pulverizing, or sawing tend to make negative results ambiguous also.

Much of the research effort on interior contamination has been devoted to the development of suitable methodology. Principal efforts along these lines have been carried out at the United States Public Health Service Center for Disease Control in Phoenix, Arizona, by the Food and Drug Administration group (formerly U.S.P.H.S.) in Cincinnati and by the Environmental Health group in the University of Minnesota School of Public Health. Other research along the same lines was done by the Dynamic Science Corporation, Monrovia, California, on contract from the Jet Propulsion Laboratory and by the United States Air Force School of Aerospace Medicine, Brooks Air Force Base in Texas. For the most part, these investigations consisted of seeding solid materials with known numbers of spores, then determining the percentage of recovery by various techniques. The general conclusions reached by these groups were that: 1) no one recovery method is suitable for all piece parts, 2) if a method suitable to that part is used, a reasonably high percentage of recovery can be demonstrated, and 3) dissolving the part in a nontoxic solvent yields the highest percent recovery. However, only a small percentage of parts is soluble in suitable solvents; thus various grinding and pulverization techniques must be utilized for most items.

Description of Studies

A number of studies have been carried out to evaluate shelf-items for interior contamination. The organization, literature reference and a summary of each are given below.

1. A technique for investigation of bacterial contamination inside electronic components. Protection Branch Report of Test No. 7-60, March 11, 1960. U.S. Army Protection Branch, Physical Defense Division, Fort Detrick, Maryland. D. Portner and R.K. Hoffman.

Objective: To develop techniques for assay of interior contamination in space hardware.

Method: All work was performed in an air tight plastic chamber fitted with glove ports, pass-through and inlet tubes. Components, broth blanks, and tools for breaking up piece parts were placed in the chamber and surface sterilized with ethylene oxide for eight hours. Filtered air was flushed through the chamber for 16 hours to remove the ETO. Parts were tested in pairs. One served as a control to assure external sterility. The other was "broken up, ground as well as possible" and placed in a tryptose broth blank for 4-6 days of incubation. Aliquots were then streaked on tryptose agar to confirm presence or absence of bacterial growth. Negative blanks were also seeded with one drop of a 24-hour Staphylococcus aureus culture to confirm that growth could be supported in the broth.

Result: Some 30 assorted electronic tubes, diodes, condensors, and resistors were assayed. No positive results were observed.

Author's conclusion: "No evidence of microbial contamination was demonstrated in the bacteriological tests performed. The results of one untaped control blank... showed that it was possible for ethylene oxide to penetrate under the bottle cap and into the broth in sufficient amount to inhibit growth of microorganisms present. Thus, the results cannot necessarily be relied upon as giving a true picture of the internal contamination of electronic components."

2. Investigation of bacterial contamination inside electronic components, Test I, Protection Branch Report of Test No. 19-60, April 14, 1960. U.S. Army Protection Branch, Physical Defense Division, Fort Detrick, Maryland. D. Portner and R. K. Hoffman.

Objective: To assay assorted capacitors, resistors, transistors and output transformers for internal contamination. The piece parts were shelf-items secured from Goddard Space Center.

Method: Items to be tested and sampling equipment were exposed to ethylene oxide in a glove box chamber for six hours for external decontamination. Bottles containing tryptose broth were wiped with hypochlorite solution and the rims of the caps were wrapped in electricians tape to prevent ethylene oxide penetration of the broth. This extra precaution was carried out to avoid problems previously encountered with ETO penetration of the broth.

Parts to be assayed were broken, ground as well as possible, and pieces placed in broth blanks and incubated at 37°C for at least seven days. If broth became cloudy, an aliquot was immediately streaked on tryptose agar to confirm bacterial

growth. Negative blanks were also streaked after incubation to confirm the absence of microbial contamination. Finally negative blanks were inoculated with Staphylococcus aureus to assure that the broth was capable of supporting growth.

Results: Positive results were indicated for 6 of 8 capacitors, 1 of 5 resistors, 1 of 4 transistors and 1 of 1 output transformers. Of the nine positives, in six instances only cocci were recovered. In the other three, spores, gram+ bacilli or combinations of these and cocci were recovered.

Authors' conclusion: "Even though only 30-40 individual components have been tested, it is evident that microorganisms are capable of surviving inside many types of such items."

3. Investigation of bacterial contamination inside electronic components. Test II. Protection Branch Report of Test No. 24-60, June 31, 1960. U.S. Army Protection Branch, Physical Defense Division, Fort Detrick, Maryland. D. Portner and R. K. Hoffman.

Objective: To assay various electronic components received from Jet Propulsion Laboratory in Pasadena, California for internal contamination. Some components heated for 13 1/2 hours at 125°C were included for comparison.

Method: The assay procedure was the same as that reported in (2) above except that after seven days incubation at 37°C aliquots of broth were transferred to tryptose broth and thioglycollate fluid medium respectively and incubated at 37°C for several additional days before being checked for bacterial growth. This procedure was introduced in an effort to avoid possible inhibitory effects of component debris. The thioglycollate was added for detection of anaerobic bacteria which might otherwise be missed.

Results: Two of 10 heated capacitors were positive, 5 of 22 unheated capacitors were positive. The results presented also indicate that 4 out of 6 heated resistors were positive while none of 10 unheated resistors were positive. The authors do not comment specifically on this unusual result leading one to suspect that the table may be in error.

Authors' conclusion: "The results ... show that internal bacterial contamination was present in 20-25 percent of the capacitors and resistors tested. Moreover, internal contamination was present in some electronic components which had been subjected to dry heat at 125°C for 13 1/2 hours."

4. Investigation of bacterial contamination inside electronic components. Test IV. Protection Branch Report of Test No. 13-61, May 19, 1961. U.S. Army

Protection Branch, Physical Defense Division, Fort Detrick, Maryland. D. Portner and R. K. Hoffman.

Objective: To determine whether electronic components were sterile after exposure to dry heat for 27 hours at 125°C.

Method: A total of 218 electronic components were received from Jet Propulsion Laboratory. Of these, 111 had been subjected to the heat treatment (125°C for 27 hours). Assay methodology was similar to that used in previous tests 1-3 above except that fluid thioglycollate medium was used throughout in place of tryptose broth. The reason given for the change was that the thioglycollate would propagate both aerobic and anaerobic bacteria and would also give better neutralization of inhibitory substances from the piece parts. In addition fewer Staphylococcus aureus cells were added in the last step to assure that even traces of inhibitory substances were not preventing the growth of small numbers of contaminants.

Results: All components tested (both heated and unheated) were found to be internally sterile. In addition the small numbers of Staphylococcus aureus grew in every instance indicating that inhibitory substances were not present.

Authors' conclusion: "For the first time when testing a large number of components, all were found to be internally sterile.... Moreover, none of the electronic components tested would have inhibited growth of microorganisms if they had been present since the few cells of Staphylococcus aureus introduced propagated in the medium containing an electronic component."

5. Investigation of Microbial Contamination Inside Cured Solid Propellant. Protection Branch Report of Test No. 13-62, Nov. 8, 1961. U.S. Army Protection Branch, Physical Defense Division, Fort Detrick, Maryland. D. M. Portner and R. K. Hoffman.

Objective: To determine whether Thiokol cured solid propellant has internal microbial contamination.

Method: Propellant cubes (1/2" size), scalpel, forceps, and fluid thioglycollate medium blanks were placed in a plastic chamber and exposed to ethylene oxide for six hours. The chamber was aerated for 16 hours, then cubes were individually minced, placed in medium blanks and incubated at 37°C for 7 days. Aliquots were then streaked on tryptose agar to check for microbial growth. Unminced control cubes were tested to assure that surface sterilization had been achieved. Negative samples were seeded with small numbers of Bacillus subtilis var. niger spores to ascertain that the medium was capable of supporting growth.

Results: None of the 37 cubes tested were positive. All unminced cubes were

negative for surface contaminants. However, Bacillus subtilis var. niger spores frequently failed to grow in media with minced cubes. Three-fold dilution of the aliquots overcame the inhibitory affect indicating that the cubes were bacteriostatic rather than bactericidal.

Authors' conclusion: "Although no viable microorganisms were found in the few samples tested it cannot be stated categorically that all of this particular propellant is sterile..."

6. Investigation of Bacterial Contamination Inside Solar Panel. Protection Branch Report of Test No. 20-60, April 14, 1960. U.S. Army Protection Branch, Physical Defense Division, Fort Detrick, Maryland. D. M. Portner and R. K. Hoffman.

Objective: To test for possible internal bacterial contamination in a solar panel.

Method: The solar panel consists of top and bottom sheets of aluminum bonded to a network of hexagonal aluminum cells. Each cell has "minute" holes drilled through its sides to permit escape of air in the vacuum of space. Thus there is some question as to whether contamination within the cells could be considered interior contamination.

The panel was cut into 1 3/8" squares, surface sterilized for six hours with ethylene oxide in the test chamber and aerated for 16 hours. Each square was then sawed in cross section to the hexagonal cells (each cell is 3/4 mm wide by 15 mm long) and the pieces placed in tryptose broth blanks to be incubated at 37°C.

Results: From five tests of two squares per test, nine out of ten squares showed contamination.

Authors' conclusion: "Since the squares were cut from a large piece of solar panel, it can be concluded that not only is there internal contamination in the solar panel but also it can not be sterilized with ethylene oxide gas in six hours. This is believed to be a simple diffusion problem. The gas would have to diffuse through the small holes from cell to cell to reach interior cells and six hours is apparently not enough time for this diffusion to occur."

7. "Sterilization of Electronic Components of Spacecraft", in Transactions of the 7th Symposium on Ballistic Missile and Space Technology. U.S. Air Force Academy, Colorado. Aug. 13-16, 1962. pp. 73-82. J.T. Cordaro and E. S. Wynne.

Objective: To determine the presence of natural contamination in the interior of assorted electronic components.

Method: Experiments were carried out in a germfree isolator. Components were disassembled and pulverized using a variety of tools. Details of surface sterilization and plating of the pulverized material are not given.

Results: A total of 11 of 166 components were found to be positive for interior contamination. The breakdown was as follows:

type of component	# examined	# positive
capacitor	101	9
resistors	45	0
diodes	5	0
electric tubes	5	0
relays	2	0
transformers	4	1
magnetic modulator	1	1
micropositioner	1	0
potentiometers	2	0

8. Studies for sterilization of space probe components. NASA Contractor Report CR-191 (Contract NASw-879), March, 1965. Castle Company, Rochester, New York. M.G. Koesterer.

Objective: To perform sterility tests on interiors of shelf-item capacitors, diodes, resistors, and transistors.

Method: Flexible film isolation systems were adapted for the task. Ethylene oxide (500 mg/liter) was used to sterilize the interior of the isolator and all items used in the test. Ethylene oxide was left in the chamber overnight, then flushed with filtered air. The item to be tested was paired with a control to determine if surface sterilization had been accomplished. The isolator interior, gloves, etc., were also tested for sterility. The component to be tested was broken into the "smallest size possible" (method and size not specified) and placed in a blank of trypticase soy broth. The broth was incubated for two weeks at 32°C. After two weeks, aliquots were streaked on tryptone glucose agar slants and incubated for two additional weeks. Negative tubes were inoculated with 100 Bacillus subtilis var. niger spores and subcultured to determine their capability of supporting microbial growth.

Results: Results were reported as positive when there was definite growth on direct or diluted culture and no doubt existed regarding either presence of contamination in the component or absence of contamination in the test system not attributed to the component.

Type of component	# tested	# contaminated
capacitors	20	2
diodes	16	0
resistors	11	2
transistors	21	2
total	68	6 = 8.8%

Author's conclusion: "These data indicate that only a small percentage of components tested contained viable microorganisms entrapped within them. Furthermore, the estimates of the levels of contaminants in these components appear to be of a low order."

9. Planetary Quarantine Unit, Quarterly Progress Report No. 17, January-March, 1967. CDC, Phoenix Field Station, Phoenix, Arizona. M. S. Favero.

Objective: To assay two toroidal transformers for both external and internal microbial contamination.

Method: No details of the method are included in the report.

Results: Both transformers were negative for internal contamination.

Author's conclusion: "No viable internal contaminants were detected."

10. Planetary Quarantine Unit, Quarterly Progress Report No. 28, October-December, 1969. CDC, Phoenix Field Station, Phoenix, Arizona. M. S. Favero.

Objective: To assay circuit boards and resistors for internal contamination.

Method: 53 fiberglass circuit boards were obtained from a radio supply house in Washington, D.C. A total of 145 resistors were removed from the circuit boards for separate assay. The resistors were measured with a micrometer, surface decontaminated with peracetic acid, then aseptically broken in half and placed in tubes containing 25 ml of Trypticase Soy Broth (TSB). The circuit boards were cut into strips approximately 1 1/2" x 1/2". They were also surface decontaminated (presumably with peracetic acid), then aseptically broken in half and placed in tubes containing 25 ml of TSB. All tubes were incubated for 30 days at 32°C and observed for growth. The total fractured area was measured for all parts tested. Later some resistors were broken again and re-incubated. (Quarterly Report No. 30, April-June, 1970).

Results: This method did not attempt to assay totally the interior of the parts as only a small portion of the interior was exposed for each part. In the initial experiment one out of 145 resistors was positive (Bacillus species) and none of the 53 circuit boards was positive. Upon refracture, none of the 68 resistors so tested was positive.

Author's conclusion: "Only one tube showed growth which was identified as a Bacillus species."

11. Methodology of Measuring Internal Contamination of Spacecraft Hardware. University of Minnesota Space Science Center Progress Report. NASA Grant NGR-24-005-063, June, 1968, pp. 19-25. V. W. Greene and B. Walker.

Objective: To ascertain the interior bio-load of a Ranger electronic module.

Method: The basic procedure utilized for this assay was termed a "disarticulation-elution-pulverization" process. The concept was that in a complex module contaminants could conceivably be located on exterior surfaces and also on other surfaces as they were stripped away and disassembled. Much of this contamination is currently categorized as "mated surface" contamination. Only in the last step were actual interior surfaces exposed by the pulverization process.

The assay was carried out in a laminar flow hood using aseptic technique and a chilled module (using liquid nitrogen). It was found that 70% of the disassembled parts could be pulverized in a Pica Blender mill while fewer than 2% were soluble in non-germicidal solvents(benzene, ether or acetone). Specific culturing techniques are not described for the pulverized powder. The authors state that "the contamination 'noise' level might be quite significant compared to actual concentration of interior contaminants."

Results: The pulverized parts were not evaluated individually. Apparently the powder was weighed and the only result reported is an average count of seven colonies per gram of material.

Author's conclusion: "...the data must be qualified by the very real possibility of contamination contributed by the technique."

12. Microbial Cell Recovery from Solid Materials. Final Summary Report, Contract No. 950740 from Jet Propulsion Laboratory, May, 1968. Dynamic Science Corporation, Monrovia, California. J. Opfell.

Objective: To assay two types of solid propellant fuel(one containing aluminum and one without) and Pfaudler-pot fuel mix for naturally occurring buried contamination.

Method: The specimens (2 1/2" x 3" blocks) were assembled in hermetically sealed polyethylene bags. Ethylene oxide solution within the bags was utilized for sterilization of tools and the exterior surfaces of the solid propellant fuel blocks.

Interior sampling was carried out by dry file abrasion of the blocks, resulting in particles of less than 1 mm in diameter. Approximately one gram powder was added to 100 ml of sterile fluid thioglycollate broth. The solution was sonicated to avoid clumping of the powder. Aliquots of the broth(0.5 ml represented 5 mg of sample) were

streaked on TSA, Sabourauds dextrose agar and anaerobic agar. Plates were incubated at several temperatures (55°C, 37°C and 25°C for TSA, 25°C for Sabourauds agar and 37°C for anaerobic agar) for seven days.

Results: The Pfaudler-pot fuel mix showed no contaminants while solid propellant fuels were contaminated. Several specimens showed in excess of 10^6 viable cells per ml (which can be extrapolated to 10^8 per gram of propellant although, in the report this extrapolation is not made and quantitative interpolation of results is left quite unclear.) Contamination was not uniform throughout the blocks with some aliquots being sterile. The mean per aliquot appears to be about 10^3 (2×10^5/gram). The propellant containing aluminum produced fewer colonies than the propellant without aluminum. The authors indicate that the technique is designed for fairly heavy contamination and is not sensitive for low numbers. A variety of organisms was recovered. They are not identified to species. However, most were aerobes or facultative anaerobes.

Author's conclusions: "Several varieties of microorganisms are present in the solid propellant, some to the extent of 10^6 per milliliter. Most of the easily detected species are aerobes though many facultative anaerobes are also present. Thermophiles are few.

Among the species of microorganisms found in the solid propellant were several more resistant to ethylene oxide than are spores of Bacillus subtilis var. niger under the conditions of the test.

The extent of contamination varies within a single block of cast propellant. The greatest contamination in the specimens containing aluminum powder (SY 251/7) produced fewer colonies on the assay plates than did the specimens which contained no aluminum powder."..."The absence of microbial flora in the pot fuel mix indicates that detectable microorganisms were present in numbers fewer than 10 per milliliter on the basis of the sampling statistics."

General Conclusion

Table I summarizes the results from the various studies described. All of the groups which have attempted even limited evaluation of shelf-item piece parts for interior contamination have been somewhat uncertain in their conclusions. It seems that the biggest failure has been the lack of adequate negative controls (items heated for very long times at very high temperatures, then assayed in identical fashion to shelf-item parts.) These controls were not reported by any group which reported positive results. However, negative results are also uncertain due to the harsh conditions generally necessary to pulverize parts into assayable powder.

The Phoenix technique of simple breaking to expose a small percent of interior surface provides some relief from this dilemma but has the disadvantage of exposing only a very small percentage of the total interior of the part.

Nonetheless, on the basis of the several studies carried out it is probably reasonable to assume that some small percentage of "shelf-item" piece parts does harbor small quantities of microbial spores, and it may be possible to assign a theoretical upper bound for internal contamination based on a MPN technique.

Table I

Summary of the Findings of the Several Groups that have Investigated Internal Contamination of Space Hardware

Investigators	Date of Report	Items Investigated		# positive /# tested
U.S. Army Protection Branch, Fort Detrick D.M. Portner and R.C. Hoffman	3/60 Test 7-60	electronic tubes, diodes condensors and resistors		0/n30
	4/60 Test 19-60	assorted components	capacitors	6/8
			resistors	1/5
			transistors	1/4
			output transformers	1/1
	4/60 Test 20-60	solar panel (cut into 1 3/8" squares)		9/10 (squares)
	6/60 Test 24-60	heated(125°C for 13 1/2 hrs.) and unheated capacitors and resistors	heated capacitors	2/10
			unheated capacitors	5/22
			heated resistors	4/6
			unheated resistors	0/10
	5/61 Test 13-61	heated(125°C for 27 hours) and unheated electonic components	heated	0/111
			unheated	0/107
	11/61 Test 13-62	solid propellant fuel (cut into 1/2" cubes)		0/37 cubes
U. S. Air Force Academy J.T. Cordaro and E.S. Wynne		assorted components	capacitors	9/101
			resistors	0/45
			electronic tubes	0/5
			relays	0/2
			transformers	1/4
			magnetic modulator	1/1
			micropositioner	0/1
			potentiometer	0/2

Castle Co. M.G. Koesterer	3/65	assorted components	capacitors diodes resistors transistors	0/20 0/16 2/11 2/21
U.S.P.H.S. CDC Phoenix Field Station M.S. Favero	3/67	toroidal transformers		0/2
U.S.P.H.S. CDC Phoenix Field Station M.S. Favero	12/69	parts broken in half only	resistors circuit boards	1/145 0/53
Univ. of Minnesota School of Public Health V.W. Greene & B. Walker	6/68	Ranger electronic module		7 coi/gram (individual parts not tested)
Dynamic Science Corp. J. Opfell	5/68	solid propellant fuel Pfaudler-pot fuel mix		$z \times 10^5$/gram sterile

APPENDIX C: ESTIMATION OF PARTICULATE LOADS ON COMPONENTS OF DEVICES MANUFACTURED IN CLEAN ROOMS

As part of the NASA planetary quarantine program a number of studies have been carried out to determine viable particles on space hardware or fall out strips located in clean rooms. Recent NASA reported studies suggest that those organisms on space hardware which are the most resistant to dry heat will probably be associated with soil particles.

Following discussions with Dr. Donald Fox, NASA Sterility Control Officer, concerning the possible importance of particulate matter in the sterilization of space hardware, a hypothetical situation was developed which we believe contains the essential conditions that will exist during the assembly of a planetary lander. This situation was presented to Roger de Roos who is a graduate engineer with an M.S. in Environmental Health. He served two years with the Division of Research Services in the National Institutes of Health and his training and experience make him qualified to make this analysis. The problem is stated below and followed by his analysis of the probable particulate load on an object (representative of a planetary lander) assembled in a clean room or tent.

Statement of the Problem

Consider that you have been called in as an environmental engineering consultant to Company A that is responsible for manufacturing the item described below. You are to make recommendations and then defend these recommendations before an expert committee made up of members from both Company A and Company B.

Company A has been assigned the job on a cost plus fixed fee basis to manufacture a complicated and delicate device for Company B. Company B has found that the performance of the stated device is influenced by the number and size of particles remaining on and in the apparatus.

The manufacturing specifications call for the apparatus to be assembled in either a class 100 clean room or a class 100 clean tent. Periodically during the manufacturing cycle, the device will be washed down with a chemical solvent. The personnel carrying out the assembly and testing program will be working on this apparatus for 8-12 months. During this time the personnel will follow the practices specified for personnel working in a clean room. The performance of all operations including the personal practices relating to clean room garments, etc. will be under continuous scrutiny of the quality assurance group.

The final unit will be assembled in a hermetic container which is approximately 4 x 10 x 10 feet. Critical dust particles will be present in two locations.

Location I will be on surfaces inside the canister which are in contact with the gas used to pressurize the canister. The particles on surfaces will be free to move with the convection currents in the canister or they may be held in place by electrostatic forces and lie flat on the surface on which they are originally deposited. A second type of surface will be those areas between devices, boxes and components that are bolted together. These will be called mated surfaces and will be cleaned with solvent about 48 hours before assembly. Company A wants to have an estimate in advance of manufacture of the concentration of dirt particles they can expect per square foot of surface in contact with the gas in the canister and per square foot of mated surface area. They require an estimate of both the number of particles per unit area and a frequency distribution of particle size. Specifically, the assignment is to prepare these estimates in a form that can be submitted to the management of Company A.

Introduction

A method for solving this problem is presented in the following paragraphs. Several references were used to help define the size distribution and number of particulates present in the air and on surfaces, and to help in defining a logical approach to the problem.

Several of the assumptions which were made in the solution of this problem need to be confirmed by experimentation in order to increase the reliability of the final estimates. Some of these experiments are listed below and discussed in greater detail in the presentation of the solution to the problem.

1. The numbers and size distribution of particles on components brought into the clean room should be determined.

2. Experiments should be conducted with solvent washes to determine the percent effectiveness of the solvent wash system for various components and sizes of particles on each component.

3. Experiments need to be conducted to determine the proportion of particles in the clean room air which are intercepted by a component and retained on the surface of the component. (Refer to the R-value in equation 1.)

4. Experiments should be conducted to determine the size distribution and numbers of particles generated during various operations of personnel in the clean room and the effects on contamination of components. This includes particulate matter contributed to components by direct contact of personnel and tools, and particulates which are contributed via the air following

generation by human activity and mechanical equipment. In this regard observations also need to be made with regard to the frequency of these operations.

Solution

The assumption is made that particles less than 0.5μ remain suspended in the clean room air and are filtered out. According to Useller in Clean Room Technology (NASA SP-5074), one cannot be assured that particles larger than 10μ are removed from the air, but rather tend to settle out in the room. Once the particles have settled out, the moving air stream will not re-entrain them. Also, the assumption is made that the class 100 clean room is a vertical flow room, taking advantage of gravity for removal of larger particles.

For particles in the air in class 100 clean rooms the distribution curve from Federal Standard 209 (Figure 1) was used. This should give a final answer somewhat lower. Particle counts in the standard are based on normal work activity periods at locations where the air approaches the work surface.

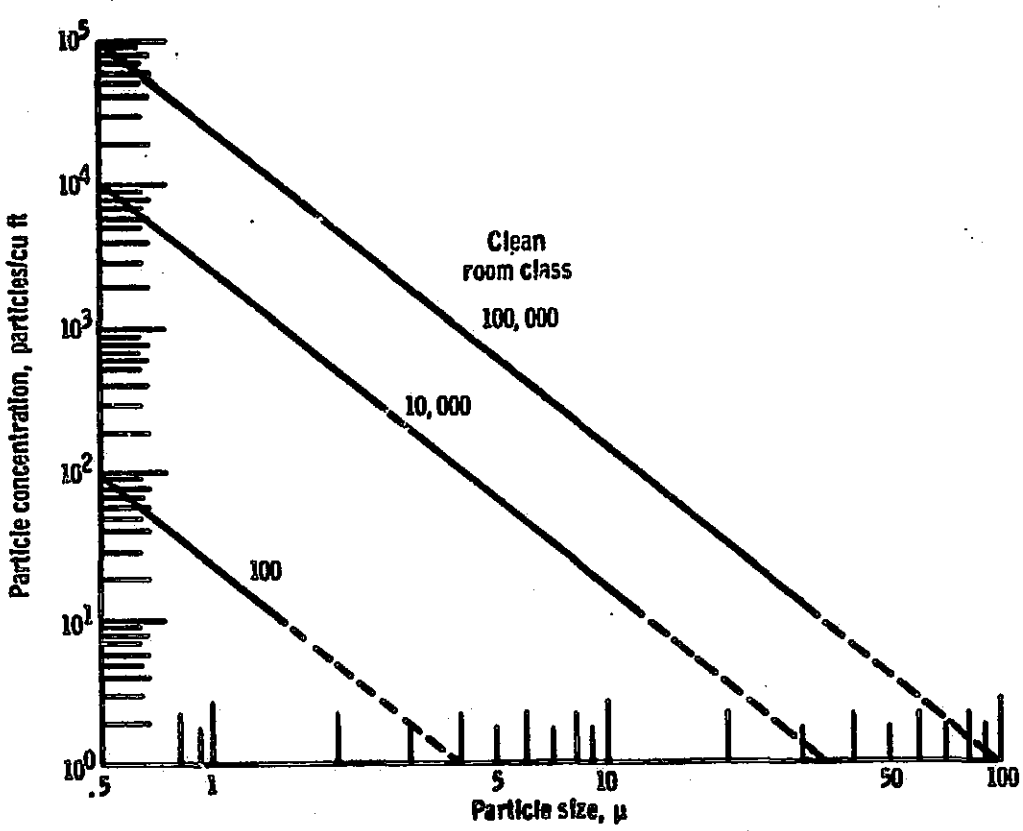


Figure 1 - Statistical particle size distribution in clean rooms

In making this estimate, one should perform some experiments in the laboratory. First, "A Microscopic Method for Measurement of Particulate Contamination" (Journal of American Association for Contamination Control, April, 1970, Robert G. Quick) should be used as a guide for experiments to determine the level

and size distribution of particulate contaminants on components brought into the clean room. Also, some experiments on components with solvent washes of various types should be performed to determine an effective solvent, and also to determine the percent effectiveness for various sizes of particles. During these tests the particles would be tabulated according to the following five size ranges: 0.5-5μ, 15-25μ, 25-50μ, 50-100μ, and greater than 100μ.

For the purpose of illustration, some assumptions will be made as to the initial load of particulate material and the effectiveness of the solvent washing method. The selection of the solvent also depends on several other factors as shown in the following table taken from "Engineering Cleaning Systems", Journal of American Association for Contamination Control, May, 1970, p. 15, written by C. B. Jacobson.

Table I

Considerations for Analysis of Cleaning System Requirements

FACILITY	CLEANING AGENT		EQUIPMENT			
Environment	Reason for Cleaning	Soil to be Removed	Materials of Construction	Configuration	Work Load	Method or Technique
Outdoors Factory Laboratory Glove Box Fume Hood Clean Bench Clean Room	Function Reliability Surface Prep. Maintenance Appearance	ORGANIC Oil Grease Wax Microbial INORGANIC Salts Compounds Rust Scale Fines	Nonferrous metal Ferrous metal Alloys Plastics Elastomers Glass Paints Inks Combinations	Flat Surface Convolutions Blind holes Material Mass Fasteners	Part Size Quantity Types of Soil Manual Automated	Dip Soak Flush Wipe Spray Electrolysis Vapor Degreasing Combinations Monitoring

The particle size distribution for the original load of particulate matter on the parts was taken from Figure 2 which was used for arriving at initial particulate load and to give some degree of reasonableness to the assumed loading.

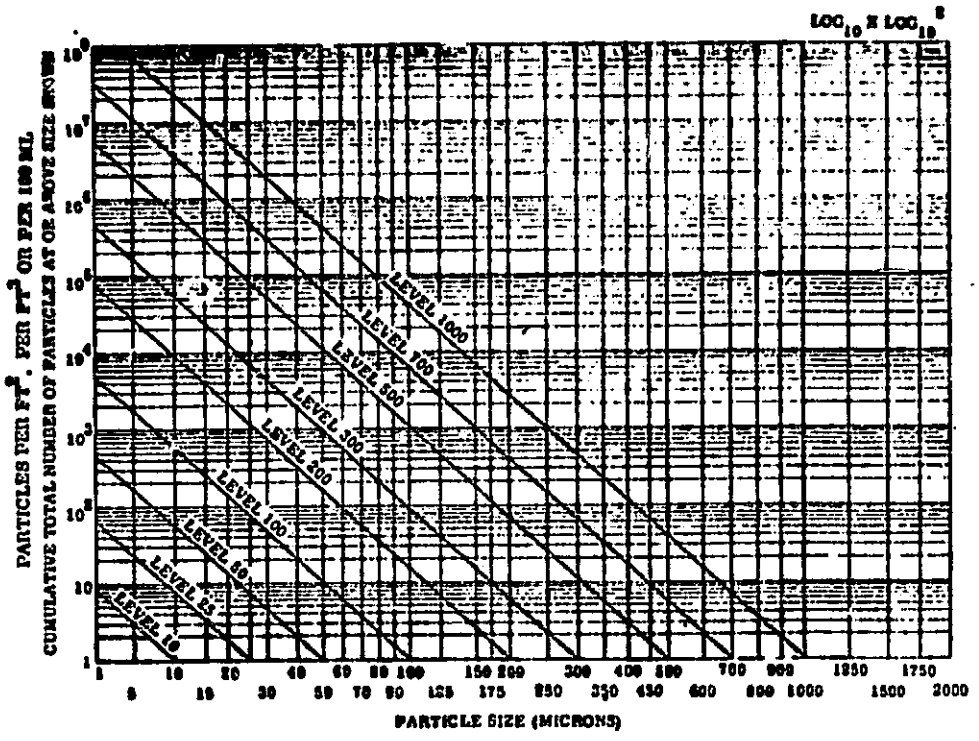


Figure 2 - Distribution of Particle and Size Count

The above figure was taken from "A Microscopic Method for Measurement of Particulate Contamination," in Journal of American Association of Contamination Control, April, 1970, p. 24, by Robert G. Quick, (original source MIL-STD-1246A).

The following table shows the size distributions (cumulative total number of particles at or above size shown) for the 1000 cleanliness level (col. 3).

Table 2

Cumulative Size Distribution Following First Solvent Wash

Ew (% for solvent flush)	size (μ)	Part/ft^2 (cumulative)	Part/ft^2 (size ranges)	Particles remaining after solvent flush [100-(1).01](4) =
95	1	20×10^7	13×10^7	6.5×10^6
97	5	7×10^7	6×10^7	1.8×10^6
97	15	1×10^7	7×10^6	2.1×10^5
99	25	3×10^6	26×10^5	2.6×10^4
99	50	4×10^5	36×10^4	3.6×10^3
	100	4×10^4	0	
99	>100	4×10^4	4×10^4	4.0×10^2

Efficiencies of solvent cleaning methods for various particle sizes are assumed. Let Ew = efficiency of solvent flush in percent. By altering the cleaning method, these percentages could be adjusted upwards or downwards to meet the requirements of the final specifications in terms of final particulate levels. The assumed percentages in Table 2 were established on the basis of larger particles being more

critical for the particular application being considered.

Following initial cleaning of the components it is necessary to estimate the amount of particulate contamination added to the parts, prior to final assembly, by air and by personnel contact. This will give an estimate of the load of particulates just prior to the final (48 hour) solvent flush. It is assumed that during the 8 to 12-month testing and assembly period, certain surfaces will be protected from deposits of particulates for a portion of the time.

First consider the amount of airborne particulate contamination added during clean room assembly. This is done by an approach similar to that presented in Section VII of Standard Operating Procedures, Experimental Clean Assembly and Sterilization Laboratory, NASA, July 1966, "Mathematical Model for Prediction of Microbiological Contamination on Spacecraft Components."

Basically the approach consists of using the following formula:

$$Ii = Pr\ V\ Ai\ R(1-K)\ Ti \qquad (1)$$

1. Ai = average area in square feet of ith component normal to direction of laminar air flow during period of time component is exposed to laminar air flow. (Use 1 ft^2 and assume component is normal to air flow).

2. V = Laminar air flow velocity (fpm) (Use 100 fpm)

3. Pr = Concentration of particles of a particular size range/ft^3

   a) Use Figure 2 on page 57 for particles in air, 100 part/ft^3 - 0.5-5μ

   b) Assume zero parts/ft^3 5μ in air moving toward components

4. K = decimal percentage of organisms which die-off. In this instance we are not concerned whether particles are viable or non-viable (k=0).

5. R = Proportion of particulates intercepted by component which are retained by component. This is another instance where it would be advisable to do experiments with the component being assembled to make estimates for R. This proportion may be affected by such factors as the shape of surface, humidity in the air, texture of the surface etc. In this instance it is assumed that the necessary experiments have been performed, and the value of R for all components was found to be 0.001. A rather low value was assumed because it is presumed that most of the particles will pass around the components in the air streams.

6. Ti = This formula is used twice: for exposure time prior to the 48-hour solvent wash and exposure following the 48-hour solvent wash. During the first 8-12 months it is assumed that components, after the initial solvent wash, are placed in a protected enclosure, unexposed to the continuous airstream as long as possible. Therefore, an average value for Ti of less than 8 months is selected. Ti = 10 days

(240 hours) for mated surfaces and Ti = 50 days (1200 hours) for other surfaces.

The original statement of the problem indicates that mated surfaces will be cleaned with solvent about 48 hours before assembly. The time of exposure to the air will be cut down to about 1 hour by covering the components between time of solvent wash and assembly. Comparing calculations a and b in Table 3 shows how minor change has a large effect on final particle count on mated surfaces.

7. Ii = number of particles added to surface of components during Ti (Remember Ai = 1 ft^2 so result is in particles per square ft.)

Table 3

Air Burden

a) 48 hrs = 2880 min Ii = Pr V Ai R(1-K) Ti = (100 part/ft^3)(100 ft/min)(1 ft^2)(1 x 10^{-3})(1-0)(2880 min) = 10 x 2880 = 28,800 particles/ft^2
b) 1 hr = 60 min 10(60) = 600 particles/ft^2
c) 10 days = 14,400 min 10(14,400) = 144,000 particles/ft^2
d) 50 = 72,000 min 10(72,000) = 720,000 particles/ft^2

The above analysis pertains to the amount of particles deposited on the surface by the air. Particles will also be deposited as a result of handling of the components during assembly. The formula shown below was used for the assessment of this particulate loading.

b = particles deposited/ft^2

$$b = \frac{\text{contacts}}{ft^2 \text{ surface area}} \times \frac{\text{contact area}}{\text{contact}} \times \frac{\text{particles}}{\text{unit contact area}} \qquad (2)$$

In an article entitled, "Particles, Particles, Particles" in Heating, Piping and Air Conditioning, January, 1963, it is indicated that particles from skin range from 10 to 300μ and metallic particles from components and tools range from 5-25μ. It is assumed that there are 20 human contacts/ft^2 and 15 tool contacts/ft^2. Since the author was unable to find any information on the specific cumulative distributions of particles deposited in each of these instances, values for numbers

of particles in various size ranges were assumed.

Table 4

Particles from Human and Tool Contact

Size μ	*Human contact part/in^2	**Tools part/in^2	b_1 Human part/ft^2	b_2 Tools part/ft^2	$b_1 + b_2$
1-5	10		2000		2000
5-15	10	10	2000	150	2150
15-25	6	10	1200	150	1350
25-50	4		800		800
50-100	2		400		400
>100	1		200		200

* Use 10 in^2/contact, 20 contacts/ft^2

** Use 1 in^2/contact, 15 contacts/ft^2

Table 5

Particles on Mated Surfaces Prior to Final Flush

Size μ	Initial burden col. 5, Table 2	10-day air burden Table 3, item 3	Contact burden Table 4	Total part/ft^2
0.5-5	6.5×10^6	1.44×10^5	2000	6.64×10^6
5-15	1.8×10^6		2150	1.80×10^6
15-25	2.1×10^5		1350	2.12×10^5
25-50	2.6×10^4		800	2.68×10^4
50-100	3.6×10^3		400	4.00×10^3
>100	4.0×10^2		200	6.00×10^2

Assuming that the final flush is 99.9% efficient for all particle sizes, Table 6 shows the particulate loading on mated surfaces following the final solvent flush.

Table 6

Particulate Concentration on Mated Surfaces Following the Final Solvent Flush

size range μ	part/ft^2 before flush	part/ft^2 after flush
0.5-5	6.64×10^6	6.64×10^3
5-15	1.80×10^6	1.80×10^3
15-25	2.12×10^5	2.12×10^2
25-50	2.68×10^4	2.68×10^1
50-100	4.00×10^3	4.00
100	6.00×10^2	6.00×10^{-1}

It is assumed that following the final solvent flush (Table 6) there is no further contact of humans or tools with mated surfaces. The only additional particulate burden will be from the air (see Table 3, Item 2), which amounts to 600 part/ft^2 for particles of 0.5 to 5μ size. Column 2 of Table 7 includes this particulate burden. Column 3 gives the particles/ft^2 on the mated surface, which is twice the number on each surface (column 2).

Table 7

Final Size Distribution and Numbers of Particles on Mated Surfaces

Size μ	Each surface	Mated surface 2 x col. 2	*Cumulative total
0.5-5	7.24×10^3	1.45×10^4	18,366.8
5-15	1.80×10^3	3.60×10^3	3,886.8
15-25	2.12×10^2	2.24×10^2	286.8
25-50	2.68×10^1	5.36×10^1	62.8
50-100	4.00	8.00	9.20
100	6.00×10^{-1}	1.20	1.20

In accordance with Figure 1, the cleanliness level is less than 200, closer to 100, for mated surfaces.

As noted previously, it is assumed that the surfaces in contact with gas in a canister can be protected from contamination for a portion of the 8-12 months they are in the room. Thus, they will only remain uncovered for 50 days.

Table 8

Particles on Exposed Surfaces Prior to Containerization

Size μ	Initial burden (Table 2)	Air burden Table 3, item 4	Contact burden Table 4, $b_1 + b_2$	Total	*Cumulative totals
0.5-5	6.5×10^6	7.2×10	2000	7.22×10^6	9.32×10^6
5-15	1.8×10^6		2150	1.86×10^6	2.10×10^6
15-25	2.1×10^5		1350	2.11×10^5	2.38×10^5
25-50	2.6×10^4		800	2.68×10^4	2.73×10^4
50-100	3.6×10^3		400	4.00×10^3	4.60×10^3
>100	4.0×10^2		200	6.00×10^2	6.00×10^2

*Cumulative total to smallest of size range given in column 1.

The assembled unit is placed in a 4 x 10 x 10 foot hermetically sealed box. The box contains a volume of 400 ft^3 of air from the laminar flow room. It is assumed that the container remains in an upright position (one 4 x 10 foot surface on the floor).

The lowest surface on the components in the hermetically sealed container

is two feet from the floor of the container. It is assumed that particles from the eight cubic feet of air in the container above the surface land on one square foot of surface on the bottom components, that is, the particles which do not remain suspended in the air.

Settling velocities may be calculated using the Stokes equation as follows:

$$\mu = \frac{2\ g\ r\ \ (\rho-\rho^1)}{g} \qquad (3)$$

ρ = density of particle

ρ^1 = density of medium

μ = terminal velocity

r = radius of particle

g = gravity

λ = viscosity

(Above equation was taken from Particle Size by Richard D. Cadle, Reinhold Pub. Co., New York, 1963.)

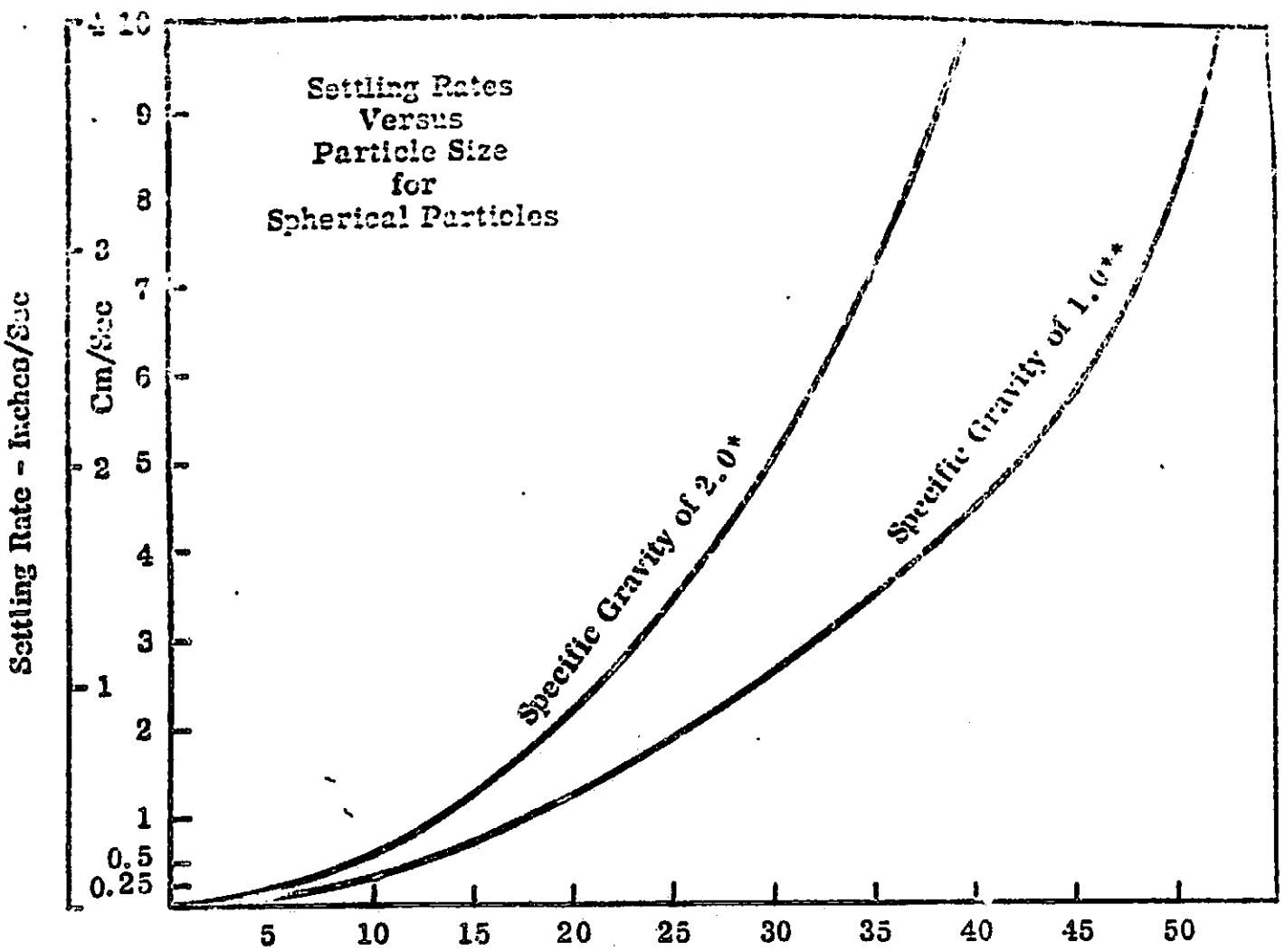
The Stokes equation is applicable for particles from 0.2 to 5μ. For particles between 2.0 and 0.1μ, settling velocity can be calculated by adding 0.04μ to the radius. Terminal velocities of particles <0.1μ are so low that it is very difficult to observe or calculate them. The following diagram (Figure 3), taken from the Handbook for Contamination Control on the Apollo Program, NASA, NHB 5300.3, August, 1966, gives a graphical solution for the Stokes equation which can be conveniently used to determine settling velocities.

Settling Velocities

From Figure 3 the terminal velocity for a 4μ particle is 9.5×10^{-2} ft/min or 11.38 ft/day and for a 0.6μ particle, 2.88 ft/day, assuming the specific gravity of the particles is 2.0.

All of the particles above 0.6μ will settle in a period of about three days. It is assumed that there will be no thermal gradients inside the container since the container itself and all materials inside were allowed to come into equilibrium with the surrounding air in the laminar flow room before the container was sealed.

Since the unit will remain in the hermetically sealed container for a period exceeding three days, the exposed surfaces eight feet from the top of the container will have collected 800 part/ft^2 ranging in size from 0.6 to 4.0μ. This is somewhat negligible, compared to the cumulative size distribution shown in the last column of Table 8 (applies to unmated surfaces). With reference to Figure 2, the final cleanliness level on unmated surfaces is less than 500.



Diameter of Particles (Microns)	Velocity of Settling**		
	Feet per Minute	Inches per Hour	Centimeters per Hour
0.1	0.00016	0.115	0.292
0.2	0.00036	0.259	0.658
0.4	0.0013	0.936	2.35
0.6	0.002	1.44	3.66
0.8	0.005	3.60	9.15
1.0	0.007	5.04	12.7
2.0	0.024	17.3	44.0
4.0	0.095	68.4	174.0

*Taken from SCTM-131-61(25) "Dust Monitoring in Clean Rooms" August 1961, by Sandia Corporation for U. S. Atomic Energy Commission. Rates are for particles in the shape of spheres having a specific gravity of 2.0.

**Compiled from "Size and Characteristics of Airborne Solids," by W. G. Frank, published in the Smithsonian Meteorological Tables. Rates are for particles in the shape of spheres, having a specific gravity of 1.0 and settling in air at a temperature of 70° F.

Figure 3 - Settling Rates for Airborne Particles

Summary

The following table summarizes the number of particles per unit area and frequency distribution of particle sizes in location 1 (surfaces in contact with a gas inside the canister) and on location 2 (mated surfaces).

Table 9

Summary of Size Distribution and Numbers of Particles

Size of Particles μ	*Mated Surfaces (part/ft^2) (Location 2)	**Exposed Surfaces (part/ft^2) (Location 1)
0.5-5.0	1.45×10^4	7.22×10^6
5.0-15.0	3.60×10^3	1.86×10^6
15-25	2.24×10^2	2.11×10^5
25-50	5.36×10^1	2.68×10^4
50-100	8.00	4.00×10^3
>100	1.20	6.00×10^2

* Cleanliness level (See Figure 2): approx. 100
** Cleanliness level (See Figure 2): approx. 500

From the initial information supplied by Companies A and B it is understood that these particulate contamination levels will meet the requirements for performance of the device as specified by Company B, and that it will be practical to manufacture the device under the conditions described in the solution to this problem. Briefly the conditions are:

1. An initial solvent wash of all components as they enter the clean room
2. Assembly of components and testing of the device in a Class 100 clean room with appropriate personnel practices.
3. Protection of components from particulates in the air in the clean room for prescribed periods of time as outlined previously in this report.
4. A final solvent wash of mated surfaces 48 hours before they are assembled and protection of these surfaces from particulates in the air for 47 of the 48 hours prior to assembly.
5. Encasement in a container which has been in the clean room for sufficient time to equalize in temperature with the clean room environment.

Should Company B decide to establish a more rigid specification on the numbers of particulates allowable on the device, it is the opinion of the author that a greater degree of cleanliness could be obtained by altering the efficiency of the solvent wash system, the number of solvent washes, and/or the assembly procedures in terms of the amount of time the components are unprotected in the clean room environment. This of course would increase the costs of assembly and testing.

APPENDIX D: SEPARATION OF A SOIL SUSPENSION OF LESS THAN 43 MICRON PARTICLE SIZE INTO <5, 5 TO 10, AND 30 TO 43 MICRON FRACTIONS

To determine if there is a relationship between the size of soil particles and the dry heat resistance of microbial spores associated with the particles it was necessary to separate soil into a number of different particle size fractions. Described below is the sedimentary method of separation that was used in our project.

Separation

The starting point in these separation studies was a soil suspension in ethanol (about 2 gm solid per 60 ml ethanol). All separations were carried out in ethanol and the final suspension was also in ethanol.

Separation of the soil suspension into fractions of 5, 5 to 10, and 30 to 43 micron particle size was carried out using the sedimentation method. Stokes Law was used to calculate the time for the different sizes of particle to fall a measured distance. Stokes Law expresses the velocity of a spherical particle falling through a liquid as a function of particle diameter. The equation for Stokes Law is:

$$V = \frac{(\rho s - \rho w)\, D^2 g}{18\, \mu} \qquad (1)$$

where V = velocity in cm/sec

ρ_s = particle density in gm/cm^3

ρ_w = fluid density in gm/cm^3

μ = fluid viscosity in gm/cm-sec

D_p = particle diameter in cm

g = gravitational constant in cm/sec^2

The separation was carried out using a 250 ml graduated cylinder. For all separations the particles were allowed to fall from the 250 ml mark to the 70 ml mark -- a distance of 18.25 cm.

The velocity in ethanol at a temperature of 22°C, when μ equals 1.158×10^{-2} gm/cm-sec, ρ_w = .787 gm/cm^3 and assuming a particle density of 2.67 gm/cm^3 is given by the following formula:

$$V = 8860\, D^2 \qquad (2)$$

Stokes Law is valid only for laminar flow around the spherical particle. The flow remains laminar as long as the Reynolds number $\left(\frac{V\, D_p \rho_w}{\mu}\right)$ is less than 0.1.

The largest Reynolds number in the suspension is 0.048 for the 43 micron particles. Therefore the flow is laminar for all the particles and Stokes Law (Equ. 1) is a valid representation for the settling velocity.

The times for the different sized particles to fall 18.25 cm through ethanol were calculated as follows:

$$\text{time} = \frac{\text{distance}}{\text{velocity}} = \frac{18.25 \text{ cm}}{8860\ D^2 \text{ cm/sec}} = \frac{2.06 \times 10^{-3}}{D^2 \text{ sec}}$$

The time in seconds for spherical particles to fall 18.25 cm as a function of diameter are tabulated below:

Particle diameter microns	Time in seconds
30	229
10	2060
5	8250
2.5	33000

The single separations were carried out as follows: A graduated 250 ml cylinder was filled to the 250 ml mark with ethanol. A 10 ml aliquot of the soil-ethanol suspension (2 gm of soil particles suspended in 60 ml of ethanol) was added to the top of the cylinder. Sedimentary separation was allowed to proceed for the period of time necessary for the particle sizes being separated. At the end of this time the suspension of particles smaller than diameter D was removed down to the 70 ml mark using a system consisting of a vacuum bottle, hose and glass tube connected to the laboratory vacuum lines. The particles in the liquid below the 70 ml mark were larger than diameter D. The sequence of steps in the single separation procedure was as follows:

A. The >30 and <43 micron diameter particles were prepared first.
   1. Ten ml of the soil-ethanol suspension of <43 micron particles were added to the top surface of the ethanol in the 250 ml graduated cylinder.
   2. 229 seconds after the addition of the sample, the >30 micron and <43 micron diameter particles were below the 70 ml mark, and the >30 micron particles were removed using the vacuum bottle and tube assembly. The ethanol plus particles removed was concentrated by centrifugation into an approximately 2 gm soil per 60 ml ethanol soil suspension to be used in the next step.
   3. The >30 micron and <43 micron diameter particles remained in the liquid below the 70 ml mark. This sample was removed and concentrated by centrifugation for further use.
B. The 10 micron diameter particles were separated second.

1. Ten ml of the soil-ethanol suspension of >30 micron diameter particles were added to the top of the ethanol in the 250 ml graduated cylinder.
2. 2060 seconds after the addition of the sample only the <10 micron diameter particles were suspended in the liquid above the 70 ml mark. These were removed with the vacuum bottle and tube assembly and concentrated by centrifugation into an approximately 2 gm soil per 60 ml ethanol soil suspension to be used in the next step.

c. The 5 micron diameter particles were separated as the third step.
1. Ten ml of the soil-ethanol suspension of <10 micron diameter particles was added to the top of the ethanol in the 250 ml graduated cylinder.
2. 8250 seconds after the addition of the sample, the >5 micron diameter and <10 micron particles were below the 70 ml mark. The <5 micron diameter particles were above the 70 ml mark and were removed using the vacuum bottle and tube assembly. Both the <5 micron particles and the >5-<10 micron particles were concentrated by centrifugation for further use.

Separation using a series of sedimentations

When the suspensions of soil particles obtained from a single sedimentation were examined, particles both larger and smaller than the design diameter range were observed. We attributed some of this variation to small particles being carried down by large particles and large particles being held up by concentration of small particles or by the wall of the cylinder. To reduce the number of particles larger or smaller than the design range we devised a sequential separation program where both the top and bottom fractions were settled three times.

The procedure followed in the sequence of three settlings for both the top and bottom fractions is shown diagrammatically in Figure 1. Briefly, the starting suspension of particles <43 micron was allowed to settle one time after which the bottom fraction was concentrated by centrifugation. The resulting sample was used as the input for a second settling; the bottom was again concentrated and used as the input sample for the third settling. The top fraction from these three settlings was composited, concentrated and then used as the input sample for the fourth settling. The top of the fourth settling was concentrated and used as the input for the fifth settling and the top of the fifth settling was concentrated and used as the input for the sixth settling. The top of the sixth settling was concentrated and constituted the <30 micron sample. The bottoms of the fourth, fifth, and sixth settlings were composited with the bottom of the third settling. This suspension was concentrated and then constituted the >30 but <43 micron sample.

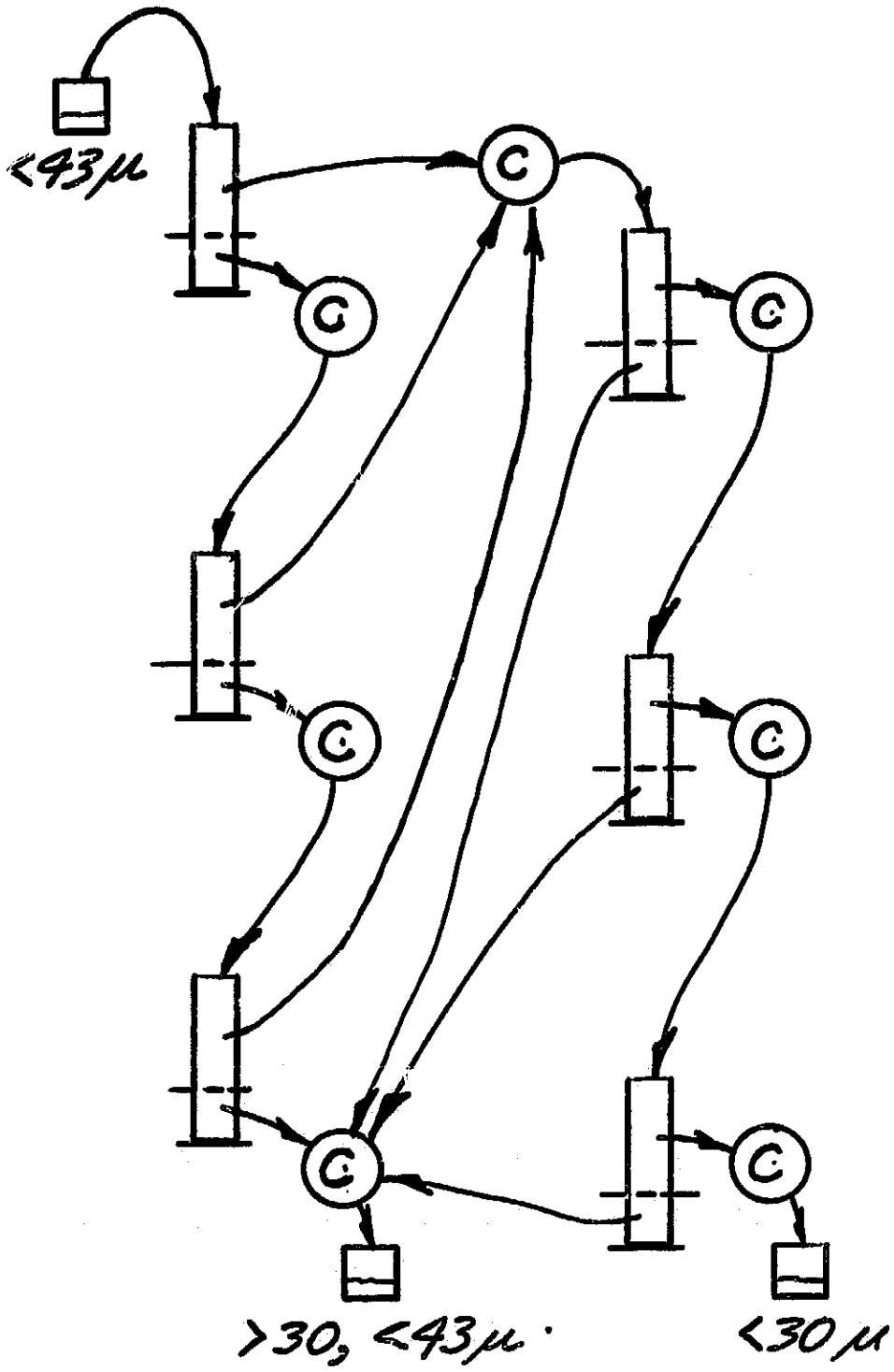


Figure 1 - Diagram showing the sequence of settlings and concentrations by centrifugation (C) for the sequential settling.

This entire procedure was repeated to obtain a <10 micron sample from the <30 micron sample. Subsequently <2.5, >2.5-<5, and >5-<10 micron soil-ethanol suspensions were obtained from the <10 micron suspension.

Discussion of the sedimentation method of separating soil suspensions into fractions of different particle size.

Calculations to arrive at particle size in sedimentation methods utilize

Stokes Law. Taylor (1948)[1] points out that the rate of fall of a large number of soil particles suspended in a fluid in a container is inaccurate because:

1. Stokes Law is based on spherical particles. In soil systems, the particles are never truly spherical; in fact, the shapes may bear little resemblance to spheres.
2. The body of water is limited. Since many particles are present, the fall of any particle is influenced by the presence of other particles. Similarly, particles near the sidewalls of the container are affected by the presence of the wall.
3. An average value for specific gravity of grains is used. The value for some particles may differ appreciably from the average value.

Our separations are subject to all the errors pointed out by Taylor, probably the most important of these errors is the fact that our soil particles were not spherical. Other sources of error are outlined below.

1. For the last particles introduced, adding 10 ml to the top of the liquid column added approximately 1 cm to the distance dropped. This represented an error of approximately 5% of the distance dropped. The time required for adding and removing the sample was approximately 10 seconds. This represented an error of approximately 5% in the worst case. The combined worst case effect of these two error sources on the particle diameter value is given by the following formula:

$$D^2 = \frac{1}{8860} \quad \frac{\text{distance } \pm 5}{\text{time } \pm 5}$$

$$D = D^2\ 1.10 \text{ or } D^2 90$$

$$= D^{105} \text{ of } D^{95}$$

$$= D \pm 5$$

The worst case effect of these two error sources on the determined diameter was 5% of the diameter.

2. Error was introduced as the result of the turbulance of adding the sample to the top of the column. The smaller particles may have been carried down faster than their sedimentary velocity. This error may be overcome by repeating the bottom portion of each run several times in order to wash out the smaller particles.

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[1] Taylor, Donald W. Fundamentals of Soil Mechanics. John Wiley & Sons, Inc., New York, 1948.

3. The temperature of the ethanol had an effect on its viscosity. The constant 8860 was computed assuming a temperature of 22°C. The change in viscosity per °C was approximately 1.7%. This represented approximately a 1% difference in the determined diameter value for each degree C.

PUBLICATIONS AND PRESENTATIONS

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